REMARKS

1. Claim Status

Method claims 39-75 remain pending in the application. Claims 39-75 were finally rejected in the September 1, 2010 Office Action. Claim 57 has been amended. Support for the amendment to claim 57 can be found throughout the originally filed specification, examples and claims and in particular, on page 3, in the second full paragraph. No new matter has been added by way of the present amendment.

Applicant respectfully requests that the method claim amendments presented herein be entered in the application as they place the application in a condition for allowance. Applicant maintains for the reasons explained below that method claims 39-75 as amended herein are allowable and should be passed to issue. Applicant addresses each of the Examiner's concerns in the sections which are presented hereinbelow.

2. Claims 57-64 Satisfy the Written Description Requirement

In the September 1, 2011 Office Action, the Examiner maintained her rejection of claims 57-64 under 35 U.S.C. § 112,¶ 1 for failure to satisfy written description on grounds that the specification of the application as originally filed, when coupled with the knowledge of those of ordinary skill in the art as of the filing date, did not support a claim to "reducing the likelihood of a recurrence of breast cancer". Applicant traverses these rejections and respectfully maintains that claims 57-64 satisfy written description for the following reasons.

Claim 57 as drafted is directed to a method of reducing the likelihood of a recurrence of estrogen-sensitive breast cancer in a patient. This clause is expressed distinctly in the specification and in particular, in the second full paragraph on page 3. It is unequivocal that the specification supports claim 57 and in particular, the use of the

language "reducing the likelihood of a recurrence of breast cancer". Apparently, this simple and incredibly straight forward concept and statement which virtually *anyone* of ordinary skill in the art could readily understand and practice somehow contravenes the written description requirement of 35 U.S.C. §112, first paragraph. Applicants respectfully traverse the Examiner's rejection for the following reasons and respectfully request that the Examiner withdraw this rejection for the reasons presented herein.

The first paragraph of 35 U.S.C. §112 requires that the specification shall contain a written description of the invention (as per the Examiner) "to clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed" (see pages 2-3 of the September 1, 2011 office action). To paraphrase, the relevant inquiry is whether the present specification describes the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. Applicant respectfully submits that he unequivocally described the invention of claim 57 (and the claims deependent thereon) in the original specification and one of ordinary skill could readily understand the description of that invention from a reading of the original application.

Claim 57 is a rather straight-forward claim which is directed to a "method of reducing the likelihood of a recurrence of estrogen-sensitive breast cancer in a patient comprising administering to said patient an effective amount of a selective estrogen receptor modulator (SERM) which has the chemical structure..." as set forth in the claim. This claim clearly and specifically is directed to a method to reduce the likelihood of the recurrence of breast cancer in a patient by administering one or more of the compounds set forth therein.

The term *recurrence of cancer* is defined rather simply by the American Society of Clinical Oncology as follows:

A "recurrence is when the cancer comes back after treatment."

The passage goes on to say: "The ultimate goal of any cancer treatment is to remove or destroy all of the cancer cells in the body. When cancer cells can no longer be found in the body, a cancer is considered to be in remission, meaning the disease is temporarily or permanently gone. A recurrence is when the cancer comes back after remission. Cancer recurs because small areas of cancer cells are difficult to find and can sometimes remain in the body after treatment. Over time, these cells may multiply and grow large enough to be found and diagnosed. Depending on the type of cancer, this can happen in weeks, months, or even many years after the primary (original) cancer was treated." See the definition and passage at the following url: **http://tinyurl.com/45mlpwb.**

A similar definition of "recurrence of breast cancer" may be found at the following url: **http://tinyurl.com/4uc7sfo .**

"After a remission of cancer, if signs or symptoms of cancer reappear, that is called a "recurrence," where cancer cells can reappear in the site of the primary tumor or can show up in a new location. Also known as a relapse, return or reappearance."

Applicant respectfully submits that the person of ordinary skill, the clinical oncologist can readily understand claims 57-64 and recognize precisely and exactly the nature of the invention which Applicant has claimed.

The Examiner's argument on page 3 of the office action with respect to biological activity of the chemically unrelated, but known SERMS tamoxifen and raloxifene (which exhibit similar activity to the compounds of the present invention) is misdirected, inasmuch as the Examiner's argument appears to be directed to the question of whether Applicant's method claims of 57-64 is effective, given the prophylactic nature of the claim, not whether or not Applicant described the rather simple method of reducing the likelihood of a breast cancer recurrence by administering a compound which is described quite clearly in the claim. To that end, Applicant respectfully submits two peer-reviewed

and published papers by Applicant Professor Richard Hochberg, *J. Clin. Endocrinol. Metab.*, July 2004, 89, pp. 3527-3535 and *J. Med. Chem.*, 2005, 48, 1428-1447 (copies enclosed), which clearly evidence that the steroidal compounds which are set forth in the claims of the present application exhibit unequivocal Selective Estrogen Receptor Modulation (SERM) activity (i.e., estrogenic activity in the liver and skeleton and anti-estrogenic activity in the uterus and breast tissue) similar to the activity which is exhibited by the clinically used SERMs tamoxiphen and raloxifene and may be used analogously.

In the present application, Applicant clearly provides sufficient description of the invention to convey to those of ordinary skill (a skilled oncologist treating breast cancer patients) that the compounds as claimed could be used in a method to inhibit or reduce the likelihood that breast cancer will recur in a patient. Given the years of experience of practitioners with compounds of similar activity such as tamoxifen and/or ramoxifene in reducing the likelihood of breast cancer recurrence using these SERM agents and the wealth of experience of clinical oncologists in treating breast cancer, a method of using the steroidal SERMs of the present invention to reduce the likelihood of breast cancer recurrence in a patient is fully described and adequately conveyed. This is further supported by the previously submitted declaration of Dr. Richard Hochberg, in paragraph 14.

Accordingly, Applicant maintains that claims 57-64 clearly satisfy the written description requirement and respectfully request the Examiner withdraw her rejection of claims 57-64 on these grounds.

3. <u>Claims 39-75 Are Nonobvious Over the Teachings of van den Broek</u>

In the September 1, 2010 Office Action, the Examiner had rejected of claims 39-75 under 35 U.S.C. § 103(a) as being unpatentable for obviousness over U.S. Patent No. 3,972,906 ("*van den Broek*").

According to the Examiner, at the time of the invention of the pending claims, the use of *van den Broek's* estrogenic compounds to treat menopausal symptoms and breast cancer would have been obvious. Essentially, the Examiner argues that "the issue is not whether the art recognized the differences in the action of the compounds in different target organs. The issue is whether the art teaches or suggests the use of the compounds as claimed by the instant invention."

Applicant agrees with the Examiner in her characterization of the issue of obviousness/non-obviousness to be resolved, but notes that Van den Broek's teachings are such that they would not given rise to the currently claimed methods and are, in fact, incompatible with the *method* claims of the present invention. This is based upon Applicant's discovery that the compounds which are set forth in the present *method* claims exhibit unexpected selective estrogen receptor modulating (SERM) activity which are used in methods which make use of that unexpected activity *to the exclusion of the activity which is taught by* ***van den Broeck.*** Thus, the presently claimed methods are directed to uses of compounds which rely on the activity which could not be surmised or determined from the teachings of van den Broeck. It is Applicant's view that the currently claimed methods are non-obvious over the teachings of van den Broeck.

Thus, Applicant respectfully contends that the compounds set forth in the present *method* claims evidence unexpected activity as selective estrogen receptor modulators (SERMs) and this unexpected activity, which was completely unrecognized by and non-obvious over the teachings of van den Broek, results claimed methods which are clearly non-obvious, patentable and distinguishable over the teachings of van den Broek, which issued in 1976[1].

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[1] Applicant points out the issue date of van den Broek only to emphasize the point that if van den Broek had taught or suggested the present invention as the Examiner argues, surely one of ordinary skill would have discovered the highly desirable SERM activity of the putatively disclosed compounds of van den Broek well before Applicant's invention in 2002, some *twenty-six* years later.

Applicant's claimed methods represent the first use of steroidal SERM's:
(a) to treat the menopausal symptoms as claimed in a patient while reducing the risk that the patient develops, or experiences a recurrence of, an estrogen-sensitive cancer;
(b) to treat an estrogen-sensitive cancer;
(c) to reduce the likelihood of a reoccurrence of breast cancer in a patient, and
(d) to treat the symptomology of menopause as claimed in a patient suffering from an estrogen-sensitive cancer.

All of the claimed methods rely on SERM activity (i.e., the unexpected combined estrogenic/anti-estrogenic activity) for the claimed methods and distinguish over the teachings of van den Broek based upon the unexpected SERM activity. Contrary to the Examiner's contention, the skilled practitioner, based upon the teachings of van den Broeck would not have recognized the activity of the present compounds as SERMS, and would, therefore, not use the present compounds (even if taught by van den Broeck) in the methods of the present invention.

At the time of the invention of the pending claims, the non-steroidal SERM Tamoxifen® was indicated for the treatment and prevention of breast cancer. It was recognized that post-menopausal patients treated with Tamoxifen® could benefit from a potential reduction in bone loss and cholesterol levels. Also, at the time of the invention of the present application, the use of steroidal estrogen receptor ***agonists*** such as those disclosed by the art of record (van den Broek) to treat post-menopausal symptoms were associated with an enhanced risk of breast cancer in that therapy and ***contraindicated*** for those reasons. The present invention addresses the concerns of the art with the unexpected discovery that *steroidal* compounds which are presently set forth in the method claims of the present invention exhibit unexpected SERM activity.

Applicant respectfully submits that van den Broek does not teach or suggest that *any* of the compounds which are disclosed therein exhibit selective estrogen receptor modulator (SERM) activity as in the present invention. Instead, van den Broek teaches a

myriad number of compounds which exhibit a broad range of activities which include contraceptive, estrogenic, progestational, ovulation-inhibiting, gonad-inhibiting and anabolic properties. Van den Broek is primarily directed to chemical compounds and the disclosure set forth in that reference is almost exclusively devoted to chemical compounds and chemical synthesis of those compounds. There is absolutely ***no*** biological activity of *any* of the compounds which are set forth in the present claims. None of the biological activity of the presently claimed compounds is exemplified and presented in van den Broek. ***None.*** Thus, with respect to the biological activity of the present compounds putativelyi disclosed in van den Broeck, that activity must be described as, at best, *prophetic*, and more accurately, non-disclosed. That is, if one of ordinary skill wanted to identify the biological activity of a number of the compounds which are set forth in the presently claimed invention which are set putatively disclosed in van den Broeck[2], that person of ordinary skill would have to make the compounds and test the compound's activity only to find the compounds did *not* possess the activity desired (estrogen agonist activity) in the relevant assays.

With respect to the suggested estrogenic activities of the van den Broek compounds, the only activity disclosed or suggested therein which is even relevant to the question of patentability of the present invention appears in column 2, lines 28-48, where van den Broek discloses that certain 11β-substituted steroidal compounds exhibit estrogenic (agonist) properties. In particular, van den Broek cites a number of specific compounds with different pharmacophores as exhibiting estrogenic activity. However, the ***only*** specifically disclosed compounds relevant to the present invention and having a similar estradiol pharmacophore to those used in the present invention are 11*β*-methoxymethyl-ethinyl-estradiol and 11*β*-chloromethyl-ethinyl-estradiol. In particular, these estradiol compounds of van den Broek have a methoxymethyl group or a chloromethyl group at the 11β position of estradiol and an ethinyl group at the 17

[2] Applicant maintains that van den Broek does not disclose the presently claimed compounds and to the extent that one of ordinary skill might surmise that the presently claimed compounds might exhibit estrogen agonist activity, that person of ordinary skill would be disabused from such a teaching after those putative compounds were actually made and tested and exhibited anti-estrogenic activity in the relevant assays.

position of estradiol. See, van den Broek, column 2, lines 28-48. It is noted that every compound which is *specifically* disclosed by van den Broek as having estrogenic (agonist) activity has a *short-chain group* at the 11β position of the steroidal pharmacophore, i.e., a chain-length of 3 (methoxymethyl) or 2 (chloromethyl) non-hydrogen atoms, regardless of pharmacophore. None of the other compounds is specifically disclosed in van den Broek as having estrogenic activity.

The biological activity (estrogen agonist activity) suggested in van den Broek for the disclosed short-chain 11β substituents (methoxymethyl or chloromethyl) is corroborated by the experiments presented on pages 22-25 of the present application. Indeed, a review of the structure activity relationship related to 11β substituents of estradiol in the present application (see especially tables 1 and 2 on pages 23 and 24) evidences that the short-chain compounds which are *specifically* disclosed by van den Broek do *indeed* exhibit estrogenic activity, but when the 11β side-chain is lengthened to 5 or more non-hydrogen atoms as presently claimed, the compounds become ***anti-*** estrogenic exhibiting SERM activity, an unexpected result, and a result which stands in complete contrast to the biological activity of the compounds disclosed by van den Broek. The compounds of the present invention exhibit *anti-estrogenic* activity consistent with their activity as SERMS, not estrogen agonists, as taught and required by van den Broek. Van den Broek does not disclose or suggest the pharmacological activity (SERM) of the presently claimed compounds. Given the clear deficiency of van den Broek, van den Broek clearly does not disclose or suggest the methods of the present invention which rely on the unexpected (and non-disclosed by van den Broeck) SERM activity of the claimed compounds in order to practice the presently claimed methods, which are completely distinguishable from the prior art teachings of van den Broeck. Moreover, the compounds which are set forth in the present method claims would not be used as estrogen agonists, because they do not have/exhibit estrogen activity consistent with the teachings of compounds of van den Broek.

It is respectfully submitted that the estrogenic compounds which are disclosed by van den Broek are *contraindicated* for use in the presently claimed methods and consequently, van den Broeck *teaches away* from the present invention. For example, each of the methods which are presented in independent claims 39, 48, 57 and 65 rely on the unexpected SERM activity of the claimed compounds in order to effectively and favorably practice the claimed invention. Noting that estrogen agonists are *contraindicated* in patients with or at risk for estrogen-sensitive cancer such as breast cancer, and estrogen agonists *worsen,* rather than *treat,* these cancers, the present compounds, which are anti-estrogenic in those tissues where estrogen-sensitive cancers develop, provide meaningful utility and benefit in the methods of the present invention, in complete contrast to the teachings of van den Broek, which do not. Moreover, following the teachings of van den Broek would *never* result in the present invention.

The presently pending claims make use of the unexpected activity exhibited by the claimed compounds. Thus, in claim 39, which is directed to a method for treating menopause while reducing the risk that a patient will develop an estrogen-sensitive cancer, the SERM compounds as claimed are particularly useful because they are effective against estrogen-sensitive cancer, whereas the van den Broek estrogenic compounds are *contraindicated* because estrogen agonists actually *increase* the risk of estrogen-sensitive cancers. In claim 48, which is directed to treating an estrogen-sensitive cancer in a patient, treatment is favorably provided by the SERM compounds of the present application because of the unexpected anti-estrogenic activity displayed, whereas the van den Broek estrogenic compounds are *contraindicated* (see the discussion below and the previously submitted declaration of Dr. Richard Hochberg, in paragraph 12). In claim 57, which is directed to reducing the likelihood of a recurrence of breast cancer (an estrogen-sensitive cancer), the compounds of the present invention, because of their unexpected SERM activity, find favorable use, whereas, the van den Broek estrogen agonist compounds are again *contraindicated.* Likewise, in claim 65, which is directed to a method for treating menopause in a patient with an estrogen-sensitive cancer, the compounds according to the present invention exhibit favorable activity in treating the

symptomology of menopause without exacerbating estrogen-sensitive cancers (because they are estrogen *antagonists* in estrogen-sensitive tissues), whereas the van den Broek compounds are contraindicted for the method of claim 65 because of the disclosed estrogenic agonist activity, which exacerbates/worsens estrogen-sensitive cancer and is contraindicated in estrogen-sensitive cancer. The same is true for all of the remaining claims, which are dependent on claims 39, 48, 57 and 65.

Thus, contrary to the Examiner's contention, the compounds which are presented in the pending method claims exhibit unexpected activity when used in the pending methods and this unexpected activity and the pending methods are not taught or suggested by van den Broek. As explained, van den Broek actually *teaches away* from the present method claims inasmuch as the biological activity which is taught by van den Broeck is *contraindicated* in the claimed methods. There can be no greater evidence of non-obviousness over a reference than when that reference, when used within the context of its teachings, teaches something which should be avoided.

In short, the presently claimed methods deviate from the prior art precisely at the point of invention where the present invention is favorably used because of the unexpected biological activity (SERM) exhibited by the claimed compounds, whereas the compounds of the prior art are actually contraindicated. The Examiner's argument that the compounds disclosed by van den Broek would *inherently* produce the claimed methods is not credible, given that the compounds which are claimed in the present methods are *not* specifically disclosed by van den Broek, and if one were to *theoretically* make compounds according to the present invention and test those compounds in a traditional estrogen assay (see the previously submitted May 2, 2007 declaration of Richard Hochberg, and in particular at paragraph 19), that person of ordinary skill would have realized that the compounds had no art recognized estrogen agonist activity. The person of ordinary skill would have concluded that the compounds set forth in the presently claimed *method* claims, in essence, are essentially *useless* for the purposes for which estrogen agonist compounds are taught in van den Broek.

Applicants further submit that the presently claimed compounds as having SERM activity are not taught by van den Broek and one of ordinary skill would not have been motivated to make and use the present compounds in the presently claimed methods which rely on SERM activity, given the teachings of van den Broek. Applicants respectfully submit that the compounds according to the present invention, which exhibit anti-estrogenic activity in traditional estrogen receptor models (see paragraphs 14-22 of the previously submitted declaration of Dr. Richard Hochberg dated May 2, 2007, enclosed) would not have been considered useful by van den Broek for treating menopause, because menopause treatment traditionally required estrogen agonist activity in the vagina and uterus, to address vaginal dryness and hot flushes, whereas the present compounds, are anti-estrogenic in the vagina and uterus. Thus, the unexpected SERM activity which is exhibited by the present compounds stands in complete contrast to the desired activity (estrogenic agonist) of van den Broek and would not be considered appropropriate. Moreover, van den Broek does not disclose SERM activity of any of the disclosed compounds. It is certainly not obvious to use a compound whose activity is not known in a method which requires that activity.

Based on what was known in the prior art and their own knowledge, those of ordinary skill in the art at the time of the invention of the pending claims would have reasonably believed that the presently claimed SERM compounds would have failed to achieve the purposes of the van den Broek taught methods *and* separately, the purpose for which the currently claimed methods are applied, precisely because of the unknown and unexpected (SERM) activity exhibited by the presently claimed compounds. *See Takeda Chem. Indust. v. Alpharma Pty Ltd.*, 492 F.3d 1350; 2007 U.S. App. LEXIS 15349; 83 U.S.P.Q.2D (BNA) 1169 (Fed. Cir. 2007), *cert. denied*, 2008 U.S. LEXIS 3015 (U.S., Mar. 31, 2008).

Regarding the Examiner's arguments that a compound and its properties are not separable, Applicants merely point out that while that basic tenet is true, the actual

compounds used in the present invention exhibit substantially different and unexpected pharmacological activity from the compounds taught by van den Broek and the methods of the present invention make use of these activity differences between the compounds used in the present invention and the prior art taught compounds. While it is true that one cannot separate a compound from its properties, where, as here, the properties of the compounds deviate from the teachings of the prior art and Applicant makes (new) use of those newly discovered properties in a way that clearly relies on and distinguishes that unexpected activity from the teachings of the prior art, invention exists. This is well settled law. The Examiner appears to be confusing the impact of van den Broeck's teachings on compound claims in general (which are not pending) and the method claims of the present application, which rely on patentability by making use of the newly discovered activity of compounds in a way not taught or suggested by the prior art.

It is Applicant's further respectful position that the Examiner's obviousness rejection ignores both the purposes for which the claimed methods are administered and the advantages of those methods, and further presupposes knowledge on the part of skilled artisans about the nature and properties of the administered compounds that could have only been gained from Applicant's invention. Van den Boerk clearly did not disclose SERM activity for any disclosed compound, let alone compounds used in the present invention. Such a hindsight reconstruction of the prior art is legally impermissible. *See Ortho-McNeil Pharma., Inc. v. Mylan Labs, Inc.*, 520 F.3d 1358, 86 U.S.P.Q.2d 1196 (Fed. Cir. 2008) (*KSR* posits a situation with a finite, and in the context of the art, small or easily traversed, number of options that would convince an ordinarily skilled artisan of obviousness; only by impermissible hindsight could patentee's selection and modification of a compound putatively developed for a different application be found obvious in this instance).

The Examiner's reliance on the doctrine of inherency in asserting the obviousness of the present invention based upon van den Broeck is misplaced given that the person of ordinary skill would first have to recognize the inherent activity of the compounds which

are presently claimed before using the compounds in methods which rely on those inherent characteristics. The point is that van den Broeck emphasizes the desirability of *estrogen agonist* activity and requires such activity, whereas the present compounds exhibit anti-estogenic activity in precisely those assays van den Broeck relied upon to establish estrogen activity and rely on that (unrecognized by van den Broeck) anti-estrogen activity to provide the favorable results in the methods of the present invention.

Accordingly, Applicant maintains that claims 39-75 are clearly nonobvious over van den Broek.

4. Claims 39-75 Are Nonobvious Over the Teachings of van den Broek, in view of Cameron, Palkowitz and Bodor

The Examiner has rejected claims 39-75 under 35 U.S.C. §103(a) as being unpatentable over van den Broek, in view of Cameron, U.S. patent publication no. 2001/0025051 ("Cameron"), Palkowitz, U.S. patent no. 6,268,361 ("Palkowitz") and Bodor, et al., U.S. patent no. 4,617,298 ("Bodor") for the reasons which are set forth in the September 1, 2010 office action on pages 7-8. Essentially, the Examiner argues that because estrogen was known to be used to treat "estrogen-sensitive" cancer, as well as the symptoms of menopause, it would have been obvious to the skilled artisan to treat estrogen-deficiency syndromes such as menopausal symptoms, osteoporosis and estrogen-dependent cancer using the compounds as taught by van den Broek. The Examiner further argues that the treatment which is taught by van den Broek, inherently results in the presently claimed methods. Applicants respectfully traverse the Examiner's rejection for the following reasons.

The teachings of van den Broek and the failure of the prior art to recognize the existence of SERM activity in any of the compounds disclosed therein, or the benefits that SERM activity provides in relationship to the claimed methods, discussed in detail hereinabove, is referenced here. In essence, van den Broek failed to teach the unexpected

pharmacological activity of the presently claimed compounds which are used in methods according to the present invention which rely on that activity, and the known pharmacological activity as taught by van den Broek is *contraindicated* for use in the presently claimed methods. It is the clearly distinguishable and unexpected SERM activity of the compounds as presently claimed in methods which rely on and distinguish over the prior art estrogen agonist compounds based upon that unexpected activity which forms the basis and foundation of the patentability of the present invention.

Van den Broek does not disclose or suggest the present invention for the reasons which are presented hereinabove. None of Cameron, Palkowitz or Bodor, taken alone or in any combination, cures the deficiencies of van den Broek in failing to suggest the present invention. Much of the disclosure of Cameron, Palkowitz and/or Bodor is actually irrelevant to the present invention, because the claimed compounds and pharmacophores disclosed in each of those references are simply unrelated to the present invention. The more generic disclosure of those references upon which the Examiner relies, as to the use of estrogen agonists in the treatment of estrogen-sensitive cancers, actually *supports* the non-obviousness and patentability of the present invention, rather than rendering the present invention obvious. Thus, the prior art relied on by the Examiner again, *teaches away* from the present invention. Applicant notes again that the present claims are directed to *methods* which use compounds which are undisclosed by van den Broeck as possessing SERM activity and utilize the unexpected activity of these compounds to produce results which rely on that unexpected activity in complete contravention to the teachings of van den Broeck.

Cameron is directed to certain compounds for preventing breast cancer. These compounds, which are completely unrelated structurally to the present invention, are said to be useful for preventing breast cancer. The teachings of Cameron have little to do with the present invention other than to point out that estrogen agonists have been used in combination with other agents in the treatment of *prostatic* cancer (paragraph 003), which is an androgen sensitive cancer, and are *contraindicated* for use in the treatment of

estrogen-sensitive cancers, including breast and endometrial cancer (paragraph 008). Notwithstanding the Examiner's reliance on the teachings of Cameron, those teachings actually emphasize the patentability of the present invention and support Applicant's point- that compounds which have SERM activity (i.e., those used in the present invention) are favorably used in the present methods, whereas the prior art estrogen agonist compounds (such as those taught by van den Broek) are actually contraindicated for use in the present invention. Cameron teaches the person of ordinary skill to *avoid* estrogen agonists and to favorably use SERMS in breast cancer and uterine cancer, the precise support for patentability that Applicant relies on. However, Cameron suggests nothing with respect to any compound disclosed in van den Broek or any steroidal compound for that matter, and no such conclusion or inference could be drawn about the compounds used in the present invention. Cameron, contrary to rendering the present invention obvious, actually *supports* the patentability of the present invention.

Turning to the teachings of Palkowitz, this reference is relevant in that it, like Cameron, also teaches that the use of estrogen agonists in treating estrogen-sensitive (estrogen-dependent) cancers is contraindicated and to be avoided (column 2, lines 40-57). Palkowitz is otherwise related to naphthyl compounds which are completely unrelated to the chemical structures of the compounds used in the present invention. Just as Cameron could be seen by one of ordinary skill in the art as supporting Applicant's claim for patentability, rather than the Examiner's position, so too does Palkowitz support the patentability of Applicant's invention. In short, Palkowitz does nothing to obviate the deficiencies of the teachings of van den Broek and Cameron in failing to suggest the present invention.

Regarding the teachings of Bodor, this reference is directed to a number of compounds which are principally directed to certain salts of steroids having estrogenic activity which are used to enhance weight control activity. None of the compounds which are disclosed therein are related to the present invention and none of the compounds disclosed therein or the disclosure provided, even allude to the compounds

and methods of the present invention. Bodor, in the background of the invention section, does make an oblique reference to the use of estrogen compounds in the treatment of breast cancer, but otherwise does not provide any disclosure which is even relevant to the present invention. It is noted that estrogen agonists actually are contraindicated for use in the treatment of estrogen-sensitive cancers (see the previously submitted declaration of Dr. Richard Hochberg), and although estrogen agonists historically were used in combination with other agents to treat cancer, that approach has been discontinued because of the tendency of that therapy to exacerbate or worsen the estrogen-sensitive cancer. Thus, the much earlier published Bodor must also be read in conjunction with the more contemporary Cameron and Palkowitz as supporting the relevance and benefit of the present invention. Bodor, in essence, does essentially nothing to cure the deficiencies of the other art in failing to suggest the present invention.

Note that with respect to the present invention, the compounds which are presently claimed in the methods of the present invention do not exhibit favorable activity as estrogen agonists and are otherwise known as anti-estrogens. So, even if Bodor's 1986 disclosure is read in isolation (i.e., without reference to the later published Cameron and/or Palkowitz) in combination with van den Broek, and estrogen agonists are suggested for treating breast cancer (an approach which is actually counterproductive and deleterious to the breast cancer therapy outcome- see the previously submitted declaration of Richard Hochberg), the present compounds would not be used pursuant to those teachings because, as explained hereinabove, the present compounds *do not* exhibit estrogen agonist activity as called for by the Bodor treatment and as taught by van den Broek. Applying Bodor to the teachings of van den Broek would result in the avoidance of the present method to use the compounds as claimed as estrogen agonists in treating cancer (as erroneously taught by Bodor), because the compounds in the presently claimed methods would have been shown to exhibit *anti-estrogen activity* in the relevant assays, not the required (as taught by Bodor) estrogen agonist activity, thus ***avoiding*** the present methods.

The position of the Examiner that it would have been obvious to use the presently claimed SERM compounds for the treatment of estrogen-dependent cancer because the prior art teachings suggest the use of the van den Broek estrogen agonist compounds for the treatment of estrogen-sensitive cancers as taught by Cameron, Palkowitz and Bodor is simply not credible. As discussed above, the person of ordinary skill would not have recognized, from van den Broek, the SERM activity of the present invention, which is favorably used in the present invention. Whether one relies on the combined teachings of van den Broek and Bodor or the combined teachings of van den Broek and Bodor with either or both of Cameron and Palkowitz, one never provides the present invention, given that van den Broek and Bodor together rely on estrogen agonist activity which is not even present in the compounds used in the present invention. Reliance on van den Broek and Bodor, in combination with the teachings of Cameron and Palkowitz is misplaced and further confuses the teachings, given that the teachings of Cameron and Palkowitz completely contradict the teachings of Bodor and lead the person of skill away from using the estrogen agonists of van den Broek in the present invention.

In sum, the person of ordinary skill would not have used the presently claimed compounds in methods requiring estrogenic agonist activity (Bodor) because the anti-estrogenic activity of the presently claimed compounds is actually *inconsistent* with the requirement for estrogenic agonist activity as taught by van den Broek and Bodor (despite the fact that the suggested method was later shown to be ineffective and/or deleterious). If one were to rely on Cameron and Palkowitz, alone or in combination with Bodor, these references would not cure the deficiencies of van den Broek- they would actually point out the fallacy and inadequacy of using the van den Broek disclosed compounds to treat estrogen sensitive cancer as taught by Bodor. In either instance, if analyzed correctly, one of ordinary skill would not have recognized the SERM activity of the presently claimed compounds because that activity was not even obliquely mentioned by van den Broek, or any of Cameron, Palkowitz and/or Bodor. It was not until the present application that the SERM activity of the presently claimed compounds became known and the benefit of such activity in the methods of the present invention which rely

on that acitivity would have been realized.

As a separate note, contrary to the Examiner's contention, the presently claimed invention is not inherent to the disclosure of the prior art references, because, as explained, the person of ordinary skill would not have even used the presently claimed compounds because they were not suggested for use as SERMS and would not have the requisite activity (as estrogen agonists) as taught by van den Broek. The Examiner's reliance on the doctrine of inherency here is not cogent inasmuch as the doctrine of inherency requires the inevitability of the claimed method occurring as a consequence of practicing the invention which is disclosed and there is no disclosure in van den Broek or in any of Cameron, Palkowitz and/or Bodor which inevitably points to the use of the presently claimed compounds in the present methods. The use of the specifically disclosed estrogen agonist compounds of van den Broek, required by a cogent inherency analysis, would clearly not result in the present invention, given that the present compounds do not competently exhibit estrogen agonist activity in those assays relied upon by van den Broek. Contrary to the Examiner's position, Applicant has not merely identified or even used inherent aspects of methods of treatment disclosed or otherwise suggested by *van den Broek* or the other references. In particular, discovering and using unidentified synthetic steroids possessing SERM activity for the purposes for which Applicant's claimed methods are administered was not suggested by, and in fact was contrary to, the teachings of the art.

Indeed, the present methods are not even accidentally practiced by relying on the teachings of the prior art given that van den Broek and Bodor teach the requirement for estrogen agonist activity (which the presently claimed SERM compounds do not possess) and the remaining teachings of Cameron and Palkowitz teach that the use of estrogen agonists should not even be used in the first place. The Examiner's reliance on the doctrine of inherency here is respectfully, misplaced. *Cf. Rapoport v. Dement, et al.*, 254 F.3d 1053, 1059, 59 U.S.P.Q. 2d 1215 (Fed. Cir. 2001).

It is respectfully submitted that the presently claimed invention is patentable. The unexpected activity of the claimed compounds as SERMS is neither disclosed nor suggested by the art of record and this unexpected activity has been put to use in claimed methods which clearly rely on and distinguish over the art based upon this unexpected activity.

Given what was known in the prior art and their own knowledge, those of ordinary skill in the art at the time of the invention of the pending claims in the present methods would have reasonably believed that the presently claimed compounds would have failed to achieve the purposes of the prior art taught methods *and* the purpose for which the currently claimed methods are applied. *See Takeda Chem. Indust. v. Alpharma Pty Ltd.*, 492 F.3d 1350; 2007 U.S. App. LEXIS 15349; 83 U.S.P.Q.2D (BNA) 1169 (Fed. Cir. 2007), *cert. denied*, 2008 U.S. LEXIS 3015 (U.S., Mar. 31, 2008). The present invention is clearly patentable over the disclosed prior art.

As a final note regarding the Examiner's discussion of the previously submitted declaration of Dr. Richard Hochberg on page 8 of the office action, Applicant wishes to point out that the discussion of SERM activity by Dr. Hochberg related to point that the compounds which appear in the present *method* claims exhibit activity which was unknown and undisclosed by van den Broek. The Examiner continues to argue about the discovery of an unappreciated property of a prior art compound or compound as being irrelevant to patentability. While the substance of the Examiner's argument in this regard would be accurate if the claims were directed to compounds or compositions, such substance is not accurate as it relates to the presently pending ***method*** claims (no compound/composition claims are presently pending). It is well settled law that a new use of an old composition or compound is patentable where, as here, the new use is directed to a method which is patentably distinguishable over the teachings of the art. Notwithstanding the fact that Applicants strongly believe that van den Broek does not teach the present compounds, either literally or inherently, such a discussion is irrelevant to the issue which the Examiner continues to rely on to reject the present application- i.e.,

whether the present ***method*** claims are directed to a new use for an old composition/compound such that the method is non-obvious over the teachings of the cited prior art. Applicant respectfully submits that the present method claims are clearly patentable based upon this well settled concept in patent law, as presented in detail hereinabove.

For the above reasons, Applicants respectfully assert that the claims set forth in the amendment to the application of the present invention are now in compliance with 35 U.S.C. Applicants respectfully submit that the present application is now in condition for allowance and such action is earnestly solicited.

No fee is due for the presentation of the amendments made herein. A petition for an extension of time is enclosed as is authorization to debit Deposit Account 04-0838. Please charge any additional fee due or credit any overpayment to Deposit Account No. 04-0838.

If the Examiner believes that discussing the present application with the undersigned attorney may materially advance the prosecution of this application, She is cordially requested to telephone the undersigned at the telephone number listed below.

Respectfully submitted,

Coleman Sudol Sapone, P.C.

Enc.

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I hereby certify that this correspondence is being sent by facsimile transmission to Examiner Barbara Badio in Group Art Unit 1628 of the United States Patent and Trademark Office, at P.O. Box 1450 Alexandria, Virginia 22313-1450" on February 18, 2011.

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Henry D. Coleman

J. Med. Chem. **2005**, *48*, 1428−144.

Nonpolar and Short Side Chain Groups at C-11β of Estradiol Result in Antiestrogens

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We have previously found that esters of 11β-estradiol carboxylates are transformed from an estrogen into an antiestrogen when the 11β-side chain is increased in length from four to five non-hydrogen atoms ($n \geq 5$). To understand the structural requirements for this transformation and obtain metabolically stable analogues that are not susceptible to esterase cleavage, we have synthesized other compounds having an 11β-side chain composed of other functional groups: ketones, amides, ethers, and thiono esters. With the exception of amides, which bind poorly to the estrogen receptor (ER), all of these compounds exhibit antiestrogenic action when the side chain length is $n \geq 5$. Ethers ($n \geq 5$), studied in more detail, inhibit the action of estradiol with either ERα or ERβ. In rat uteri they are estrogen antagonists/weak agonists and decrease the concentration of cholesterol in blood (an hepatic estrogenic action). Thus, these short chain and nonpolar 11β-analogues of estradiol have tissue specific antiestrogenic/estrogenic actions, characteristics of selective estrogen receptor modulators.

Introduction

In the course of studies to produce locally active "soft" estrogens for the treatment of menopausal dyspareunia (vaginal dryness), we synthesized various derivatives of estradiol (E_2) in which alkyl esters of carboxylates were appended to the steroid nucleus.[1,2] These groups were attached at C-7α, C-11β, 15α, and 16α, positions in the nucleus of E_2 that are known to interfere minimally with binding to the estrogen receptor (ER).[3] Biological studies of these esters of E_2-carboxylates showed unusual activities in which esters at the same positions and with the same chain length but different alkoxy groups had disparate activities. However, the most unusual estrogenic response was observed with the methyl and ethyl esters of E_2-11β-methylcarboxylate (E11-2,1 and E11-2,2, respectively) (Figure 1). Both of these esters of the E_2-11β-carboxylate bound with high affinity to ER, with E11-2,2 the better ligand. However, while E11-2,1 caused an appropriate estrogenic simulation in the estrogen-responsive Ishikawa cell, E11-2,2 was almost inactive.[4] Subsequently, we found that E11-2,2 is an antiestrogen: it inhibits estrogen stimulation of an endogenous gene (alkaline phosphatase) in Ishikawa cells and in cells transfected with a construct of either ER subtypes, ERα or ERβ, and an estrogen response element (ERE) reporter gene construct.[5] When tested in vivo in immature rats, E11-2,2 inhibited uterotrophic stimulation with E_2. When administered alone to ovariectomized rats, E11-2,2 had minimal uterotrophic action, but it had the estrogenic effect of decreasing plasma cholesterol (upregulation of hepatic lipoprotein receptors[6]) and stimulating bone growth.

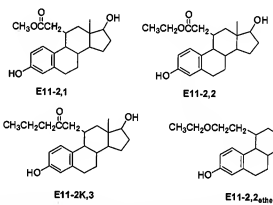

Figure 1. Examples of E_2-11β-ester, -ketone, and −ether, where the side chain has a length of five non-hydrogen atoms.

Thus, we showed that E11-2,2 is an antiestrogen in the uterus and an estrogen in liver and bone.

Compounds that have tissue-selective estrogenic/antiestrogenic actions are called selective estrogen receptor modulators, SERMs, well-known therapeutic agents, exemplified by raloxifene and tamoxifen. Their action is different from pure antiestrogens, such as ICI 164,384, that act as antiestrogens in estrogen-responsive tissues.[7] Both pure antiestrogens and SERMs, whether nonsteroidal or steroidal, share a common structural feature of a long side chain with a polar or charged substituent. This side chain interferes with the agonist-induced conformation of helix 12 in ER,[8−10] which in turn interferes with coactivator binding required for transcriptional activation of estrogen-responsive genes.[11,12] E11-2,2 has a relatively short and nonpolar side chain at the 11β-position of E_2. This side chain is dramatically different from that of all other antiestrogens or SERMs, and consequently, it appears that E11-2,2 may act with ER by another mechanism.

E11-2,1 and E11-2,2 are esters that were originally intended to act locally within the tissue (vagina) to

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‡ Both contributed equally to these studies.

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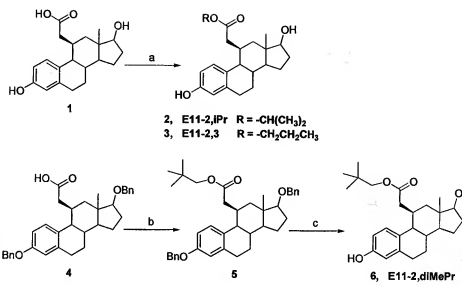
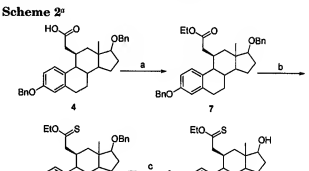
Scheme 1[a]



[a] (a) ROH, $SOCl_2$; (b) DMAP, DCC, neopentyl alcohol, CH_3CN; (c) BCl_3, CH_2Cl_2, 0 °C.

which they are applied. They were designed to be metabolically labile, e.g. substrates for esterases that would convert them to their parent carboxylates which are inactive, and thus, they would lack systemic activity. This current study was undertaken to probe what structural features of the 11β-side chain esters produce this changeover of E11-2,1 from an estrogen to E11-2,2, an antiestrogen. This transformation of an agonist into an antagonist occurs with the addition of a single methylene unit, replacing a methyl ester with an ethyl ester, thereby increasing the length of the C-11β side-chain of E_2 from four to five non-hydrogen atoms (termed the $n \geq 5$ rule). We now ask, do other analogues containing portions of the ester group (ketones and ethers) or substitutions (amides or thiono esters) also exhibit this property? We expect that antagonists resulting from this study would be considerably more metabolically stable than esters and therefore potentially therapeutically useful.

Scheme 2[a]



[a] (a) ROH, $SOCl_2$; (b) Lawessons reagent, *o*-xylene, 140 °C; (c) BCl_3, CH_2Cl_2, 0 °C.

Chemistry

The isopropyl and propyl esters [E11-2,iPr (**2**) and E11-2,3 (**3**)] were prepared by reacting **1**[2] with the appropriate alcohol in the presence of $SOCl_2$ (Scheme 1); the neopentyl ester, E11-2,5 neo **6**, was prepared by reacting the previously prepared benzyl-protected compound **4**[2] with neopentyl alcohol in the presence of DMAP and 1,3-dicyclohexylcarbodiimide. Deprotection with BCl_3 in CH_2Cl_2 at 0 °C gave the ester **6**.

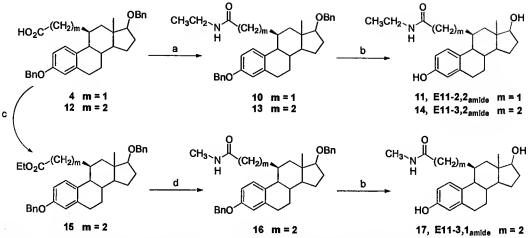
For the synthesis of the thiono ester **9**, E11-2S,2 (Scheme 2), the protected acid **4** was first esterified with ethanol in the presence of $SOCl_2$ and then thiated with Lawesson's reagent[13] in *o*-xylene at 140 °C in a sealed vial to give **8**. Deprotection as above with BCl_3 gave E11-2S,2 (**9**).

The syntheses of the E11-2 and E11-3 amide derivatives are shown in Scheme 3. The protected acid **4** or **12** was coupled[14] with ethylamine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and DMAP in CH_2Cl_2 to give **10** and **13**, respectively. These compounds were then deprotected with BCl_3 as above, giving E11-2,2_{amide} (**11**) and E11-3,2_{amide} (**14**). The methyl amide in the E11-3 series was prepared by esterification of **12** followed by aminolysis of the resulting ethyl ester **15** with 33% ethanolic methylamine catalyzed by NaCN[15,16] to give **16**. Removal of the benzyl groups with BCl_3 as above gave E11-3,1_{amide} (**17**).

The syntheses of the ketone analogues are shown in Scheme 4. Addition of ethylmagnesium bromide or propylmagnesium chloride to the previously obtained nitrile **18**[2] followed by acidic workup[17] gave the ketones **19** and **21**, respectively. Deprotection with BCl_3 as above gave E11-2K,2 (**20**) and E11-2K,3 (**22**). For the E11-3K series, hydroboration/oxidation of the 11β-vinyl steroid **23**[18] gave the terminal alcohol **24**. Tosylation of the alcohol followed by displacement with NaCN gave the nitrile **26**. Reaction of **26** with the appropriate Grignard reagent as above followed by deprotection with BCl_3 gave E11-3K,1 (**28**), E11-3K,2 (**30**), and E11-3K,3 (**32**).

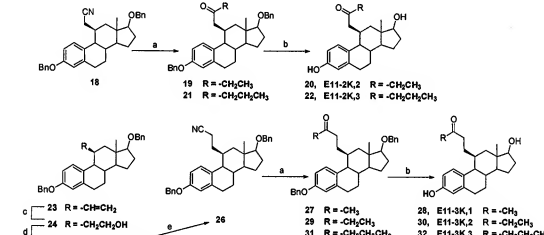
For the synthesis of E11-0,4_{ether} (**40**) (Scheme 5), the protecting group on 11β-hydroxyestrone 3-acetate[19] was replaced with a benzyl group and the 17-ketone was protected as a ketal giving **36**. Deprotonation of **36** with KH, followed by reaction with butyl *p*-toluenesulfonate[20] in toluene at 80 °C, gave the ether **37**. Removal of the ketone protecting group and stereoselective reduction of the ketone with $LiAlH_4$ in THF at −78 °C gave **39**. Hydrogenolysis of the benzyl group using 5% palladium

Scheme 3[a]



[a] (a) EDC, DMAP, $EtNH_2$, CH_2Cl_2; (b) BCl_3, CH_2Cl_2, 0 °C; (c) EtOH, $SOCl_2$, 45 °C; (d) NaCN, EtOH, CH_3NH_2.

Scheme 4[a]



[a] (a) (i) RMgX, Et_2O, (ii) 1 M HCl; (b) BCl_3, CH_2Cl_2, 0 °C; (c) (i) catechol borane, $LiBH_4$, THF, rt (ii) NaOH, H_2O_2, EtOH–H_2O; (d) TsCl, Pyr; 0 °C, (e) NaCN, DMSO, 90 °C.

on carbon in EtOH under an atmosphere of H_2 produced E11-0,4_{ether} (**40**).

The synthesis of the E11-1 ethers is shown in Scheme 6. 11β-hydroxymethyl steroid **41**[2] was deprotonated with KH and reacted with the appropriate alkyl iodide followed by deprotection with BCl_3 in CH_2Cl_2 or by hydrogenolysis giving E11-1,1_{ether} (**43**), E11-1,2_{ether} (**45**), E11-1,3_{ether} (**47**), and E11-1,4_{ether} (**49**). To construct the 2-fluoroethyl ether, the anion of **41**, generated in the presence of 18-crown-6, was reacted with (2-bromoethoxy)-*tert*-butyldimethylsilane, giving **50**. Desilylation, mesylation of the resulting alcohol, and displacement with fluoride gave the 2-fluoroethyl ether **53**. Hydrogenolysis of the benzyl groups with 5% palladium on carbon in EtOH–EtOAc under an atmosphere of H_2 gave E11-1,$2F_{ether}$ (**54**).

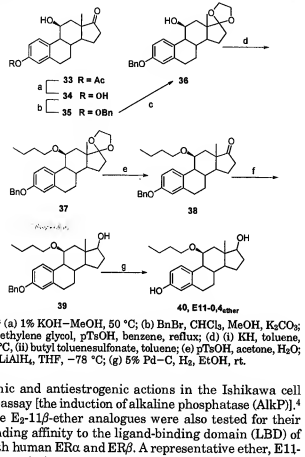
The synthesis of the E11-2 ethers is shown in Scheme 7. The anion of alcohol **24** was reacted with the appropriate alkyl iodide followed by deprotection with BCl_3 in CH_2Cl_2, giving E11-2,1_{ether} (**56**), E11-2,2_{ether} (**58**), and E11-2,3_{ether} (**60**). For the bulky or substituted ethers, alcohol **24** was tosylated and then reacted with the anion of the appropriate alcohol (generated in the presence of 18-crown-6) in toluene at 80 °C, giving the respective ether. Hydrogenolysis of the benzyl groups using 5% palladium on carbon under an atmosphere of H_2 gave E11-2,iPr_{ether} (**62**), E11-2,tBu_{ether} (**64**), and E11-2,$2F_{ether}$ (**66**).

For the preparation of the E11-3 ethers (Scheme 8) the procedure for the E11-2 ethers was used with alcohol **67**[2], giving E11-3,1_{ether} (**69**), E11-3,2_{ether} (**71**), E11-3,iPr_{ether} (**74**), E11-3,tBu_{ether} (**76**), and E11-3,$2F_{ether}$ (**78**).

Results

These studies were designed to determine whether the transformation from estrogen agonist to estrogen antagonist occurs when the length of the side chain is increased from four to five (non-hydrogen) atoms in 11β-substituted compounds containing functional groups other than esters ($n \geq 5$ rule). We synthesized various E_2-11β-side chain analogues: ethers, ketones, amides, and thiono esters (Tables 1 and 2). In addition, we synthesized three esters in the E11-2 series that are larger than E11-2,2 in order to determine whether they retain the antagonist property. These compounds were tested for their affinity to the ER by displacement assays (inhibition of the binding of [^{3}H]E_2 to ER in ovariectomized rat uterine cytosol) and for their estro-

Scheme 5[a]



[a] (a) 1% KOH−MeOH, 50 °C; (b) BnBr, $CHCl_3$, MeOH, K_2CO_3; (c) ethylene glycol, pTsOH, benzene, reflux; (d) (i) KH, toluene, 80 °C, (ii) butyl toluenesulfonate, toluene; (e) pTsOH, acetone, H_2O; (f) $LiAlH_4$, THF, −78 °C; (g) 5% Pd−C, H_2, EtOH, rt.

genic and antiestrogenic actions in the Ishikawa cell bioassay [the induction of alkaline phosphatase (AlkP)].[4] The E_2-11β-ether analogues were also tested for their binding affinity to the ligand-binding domain (LBD) of both human ERα and ERβ. A representative ether, E11-$2,2_{ether}$ (**58**), was tested for its antiestrogenic affect in cells that were transfected separately with ERα or ERβ with an ERE-luciferase reporter gene construct;[5] in addition, it was tested in vivo in rats to assesses its estrogenic/antiestrogenic action on the uterus (uterine weight) and liver (plasma cholesterol concentration).[6]

Amides. We synthesized the amides corresponding to the esters E11-2,2, E11-3,1, and E11-3,2: E11-$2,2_{amide}$ (**11**), E11-$3,1_{amide}$ (**17**), and E11-$3,2_{amide}$ (**14**), respectively (Table 1). Unlike the esters, the amides bind poorly to ER, and therefore, as would be expected, all of them are only weakly active in the Ishikawa estrogen bioassay.

Thiono Esters. We synthesized E_2 analogues in which the carbonyl oxygen of the ester was replaced with sulfur, resulting in thiono esters, compounds that are known to be resistant to hydrolysis by esterase.[21] We attempted the synthesis of two thiono esters, E11-2S,2 (**9**) and E11-3S,1. E11-3S,1, the methyl ester of 11β-(2-thiocarboxyethyl)-E_2, proved to be unstable, as it decomposed by hydrolysis to the corresponding ester 11β-(2-carboxyethyl)-E_2, E11-3,1. E11-2S,2 has a relative binding affinity (RBA) of 10% (Table 1), which is significantly lower than that of the ester E11-2,2 (45% ± 19%).[2] In the Ishikawa assay E11-2S,2 is an estrogen antagonist, inhibiting the E_2 stimulation of alkaline phosphatase (AlkP) with a K_i of ~3 nM, which is about the same as previously found for the ester E11-2,2.[5] The decrease in binding activity without an accompanying decrease in antagonism may best be explained by an increase in metabolic stability of the thiono ester.

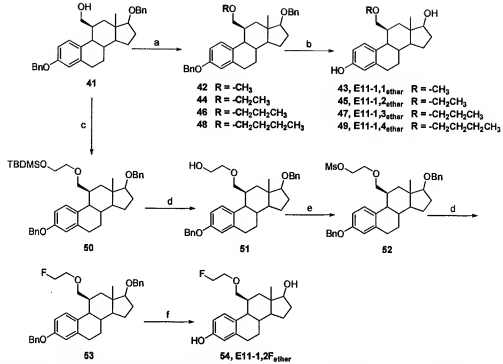
Esters. We synthesized three esters in the E11-2 series with increasing chain length and/or steric bulk, E11-2,3 (**3**), E11-2,iPr (**2**), E11-2,diMePr (**6**) (Table 1). As expected ($n \geq 5$), all of these compounds are estrogen antagonists. E11-2,3 has an RBA for the ER of 38% and a K_i in the Ishikawa assay of 1 nM compared to E11-2,2, which has an RBA of 45% and a K_i of 3.9 ± 1.4 nM.[5] While E11-2,iPr, a relative of E11-2,2 with a branched methyl group, has a lower RBA of 17%, it is a better antagonist than E11-2,2, with a K_i of 2 nM. E11-2,diMePr, a bulky relative of E11-2,3, has comparatively poor binding and inhibition with respect to E11-2,3, 10% and 5 nM, respectively. It appears in this case that the bulky 2,2-dimethylpropyl group hinders binding to the ER to an extent that causes lower inhibition.

Ketones. We synthesized a set of ketones, compounds in which the ester oxygen in the E_2-ester analogues is replaced by a methylene unit (Table 1). For example, E11-2K,2 (**20**) related in this manner to the ester E11-2,1 has a comparable RBA 18% vs 24% ± 8% for the ester.[2] Like the ester E11-2,1, which is an agonist with a relative stimulatory activity (RSA) of 16% ± 10%,[2] E11-2K,2 is also an agonist, albeit a weak one with an RSA of 1.9% (E11-2K,2 never reaches maximal stimulation and plateaus at about 50% of the maximal value produced by E_2. An example of this behavior is shown with E11-$1,2_{ether}$ in Figure 2a. E11-3K,1 (**28**) ($n = 4$) has very low binding with an RBA of 1% and is inactive in the Ishikawa assay. E11-2K,3 (**22**), analogous to the ester E11-2,2, is an antagonist with an RBA of 13% and a K_i of 3.1 nM. E11-3K,2 (**30**), an antagonist related to the ester E11-3,1, has an RBA of 3.7% and a K_i of 16 nM as compared with ester E11-3,1 with an RBA of 18 ± 3%[2] and a K_i of 10 ± 2 nM (unpublished data). E11-3K,3 (**32**), an antagonist analogous to the ester E11-3,2, has an RBA of 7.7% and a K_i of 13 nM as compared with ester E11-3,2 with an RBA of 25% ± 6%[2] and a K_i of 7 ± 4 nM (unpublished data).

These ketones fit the pattern where compounds with side chains of four non-hydrogen atoms or less exhibit agonist activity and side chains of five non-hydrogen atoms or greater exhibit antagonist activity ($n \geq 5$ rule). In general, for ketones of the same length, compounds with the ketone group at the 2′-position have better binding than compounds with the ketone group at the 3′-position. Most of these ketones have lower ER binding affinity and the same biological activity, albeit not necessarily the same potency, as their corresponding ester.

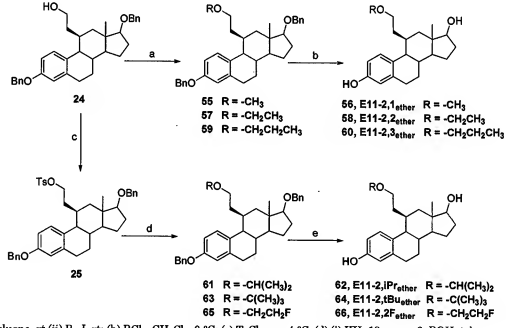
Ethers. We prepared a series of ethers, compounds in which the carbonyl unit of the ester group in the E_2-ester analogues is replaced by a methylene unit (Table 2). For example, E11-$2,1_{ether}$ (**56**), related in this manner to the ester E11-2,1, has an RBA of 25%, almost identical to that of the ester E11-2,1 (discussed above). However, it is a mixed agonist/antagonist in the Ishikawa cell. E11-$2,2_{ether}$ (**58**), an antagonist analogous to the ester E11-2,2, has an RBA of 31%, not significantly different from that of the ester, and a K_i of 0.3 nM, which is about 10 times the potency of the ester. E11-$3,1_{ether}$ (**69**), an antagonist analogous to the ester E11-3,1, has an RBA of 34% and a K_i of 0.3 nM; it is a better ligand for ER and a much more potent antagonist compared to the corresponding ester. E11-$3,2_{ether}$ (**71**), an antagonist analogous to the ester E11-3,2, has an RBA of 48% and a K_i of 0.3 nM, which is again a much

Scheme 6[a]



[a] (a) (i) KH, toluene, rt, (ii) R−I, rt; (b) BCl_3, CH_2Cl_2, 0 °C; (c) (i) KH, toluene, rt, (ii) (2-bromoethoxy)-*tert*-butyldimethylsilane, 60 °C; (d) $^nBu_4NF$, THF, (e) MsCl, pyr, 4 °C; (f) 5% Pd−C, H_2, EtOAc−EtOH, rt.

Scheme 7[a]



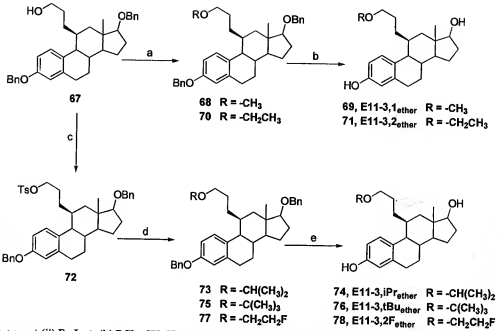
[a] (a) (i) KH, toluene, rt (ii) R−I, rt; (b) BCl_3, CH_2Cl_2, 0 °C; (c) TsCl, pyr, 4 °C; (d) (i) KH, 18-crown-6, ROH, toluene, rt (ii) **25**, 80 °C (e) 5% Pd−C, H_2, EtOAc−EtOH, rt.

more potent ER ligand and antagonist than its corresponding ester.

We synthesized a number of additional ethers to examine the effect of ether chain length in terms of the $n \geq 5$ rule, and the position of the ether oxygen on ER binding and estrogen antagonistic activity (Figure 2, Table 2). All of these ethers adhere to the rule. E11-1,1_{ether} (**43**) ($n = 3$) is a very potent agonist with an RSA of 125%. Like E11-2,1_{ether}, E11-1,2_{ether} (**45**) ($n = 4$) is an agonist/antagonist. The compounds E11-1,3_{ether} (**47**) ($n = 5$), E11-1,4_{ether} (**49**), and E11-2,3_{ether} (**60**) ($n = 6$) are all highly potent antagonists with $K_i = 0.1–0.3$ nM. We also synthesized 11β-butoxy-E_2, E11-0,4_{ether} (**40**) ($n = 5$), which binds very weakly to the ER with an RBA of 2.1% and almost no biological activity.

We tested for the effect of fluorine incorporation at the 11β-ether side chain terminus, synthesizing E11-1,$2F_{ether}$ (**54**), E11-2,$2F_{ether}$ (**66**), and E11-3,$2F_{ether}$ (**78**). The compounds E11-1,$2F_{ether}$ and E11-2,$2F_{ether}$ show modest ER binding of 10% and 16%, respectively, and

Scheme 8[a]



[a] (a) (i) KH, toluene, rt (ii) R−I, rt; (b) BCl_3, CH_2Cl_2, 0 °C; (c) TsCl, pyr, 4 °C; (d) (i) KH, 18-crown-6, ROH, toluene, rt (ii) **72**, 80 °C (e) 5% Pd–C, H_2, EtOAc–EtOH, rt.

show poor biological activity. While E11-3,2F$_{ether}$ shows good ER binding (47%), it is only a modest antagonist with a K_i of 1.1 nM.

We prepared a series of compounds in which we incorporated branched methyl groups at the end of the 11β-ether side chain to investigate the possibility that a bulky side chain terminus might enhance antiestrogenic activity in compounds with good ER binding (Table 2). The compounds are analogues of E11-2,2$_{ether}$ and E11-3,2$_{ether}$. In the case of E11-2,iPr$_{ether}$ (**62**), ER binding, RBA, is decreased by half, but the antiestrogenic activity is unaffected as compared to that of E11-2,2$_{ether}$. However, for E11-2,tBu$_{ether}$ (**64**), estrogen receptor binding is dramatically decreased as is antiestrogenic activity. Interestingly, there is a dramatic improvement in both the ER binding and in estrogen antagonism as the same modifications are made in the E11-3 ether series. Here E11-3,iPr$_{ether}$ (**74**) (RBA = 114%) has a K_i of 0.2 nM and E11-3,tBu$_{ether}$ (**76**) (RBA = 81%) has a K_i of 0.09 nM.

To determine the selectivity of the binding of the ether analogues to ERβ, we measured the binding to the LBD of human ERβ (M_{214}–Q_{530})[22] in comparison to the LBD of human ERα (M_{250}–V_{595}).[23] As can be seen in Table 2, the 11β-ethers of E_2 have little binding selectivity for ERα or ERβ. In addition, we investigated the action of a representative ether, E11-2,2$_{ether}$ (the ether analogue of the ester E11-2,2 characterized previously[2,5]), in JAR cells that had been separately transfected with ERα or ERβ with a consensus ERE linked to a luciferase reporter gene. Like the ester E11-2,2, the ether E11-2,2$_{ether}$ is an estrogen antagonist with both ER subtypes; it inhibits E_2 stimulated transcription of the reporter gene with both ERα and ERβ (Figure 3)

We investigated the possibility of a differential in vivo action of E11-2,2$_{ether}$ on the uterus and liver. Accordingly, E11-2,2$_{ether}$ was studied in vivo in the classical estrogen bioassay, determining its effect on the uterotrophic stimulation of E_2 in the immature rat. This experiment was performed four times with similar results. One such experiment is shown in Figure 4. It can be seen that E11-2,2$_{ether}$ at 100 ng has only a small stimulatory effect on uterine weight ($P < 0.01$) and this dose partially inhibits the uterotrophic stimulation of 20 ng of E_2 ($P < 0.001$). At 1 μg, E11-2,2$_{ether}$ is again slightly estrogenic ($P < 0.001$), and it also inhibits the uterotrophic stimulation of 20 ng of E_2 ($P < 0.001$). There were slight differences in the other experiments: in two of them, 100 ng of E11-2,2$_{ether}$ had no uterotrophic effect but still partially inhibited the action of E_2; in two experiments, the 1 μg dose of E11-2,2$_{ether}$ was again slightly uterotrophic but completely abolished the uterine response to E_2. In all experiments, the low dose of E11-2,2$_{ether}$ inhibits E_2 stimulation of the uterus, and the high dose inhibits even more (in some of the experiments completely). While the low dose was slightly estrogenic in some experiments, it was not in others (likely due to a relatively high SD). The high dose was slightly estrogenic in all experiments. Thus, these experiments clearly show that E11-2,2$_{ether}$ is an antagonist with weak agonist activity, a mixed agonist/antagonist, in the uterus of the rat.

We compared the action of E11-2,2$_{ether}$ on the liver and uterus by measuring its effect on plasma cholesterol level and uterine weight in the ovariectomized rat. The rats were injected with two separate doses of E11-2,2$_{ether}$: (1) 36 ng/day and (2) 180 ng/day and E_2 20 ng/day. E_2 produced both a significant increase in uterine weight and a decrease in plasma cholesterol concentration ($P < 0.01$) (Figure 5a,b). In contrast, each dose of E11-2,2$_{ether}$ had no statistical effect on uterine weight and yet both produced a dose-dependent decrease in plasma cholesterol level ($P < 0.01$). The lack of uterotrophic activity in this experiment compared to the previous one in immature rats is most probably due to the lower dose employed in this experiment with the

Table 1. Estrogenic Properties of 11β-Analogues of E_2

Compound[a]	RBA[b] %	Inhibition[c] K_i nM
 E11-2,3 (3)	38 ± 15	1.0 ± 0.6
 E11-2,iPr (2)	17 ± 4	2.1 ± 0.4
 E11-2,5DiMePr (6)	10 ± 3	5.2 ± 2.4
 E11-2S,2 (9)	10 ± 4	2.7 ± 1.3
 E11-2,2amide (11)	0.1 ± 0.1	w. ag. / ant.[d]
 E11-3,1amide (17)	0.6 ± 0.8	w. ag. / ant.[d]
 E11-3,2amide (14)	3.7 ± 2.9	w. ag. / ant.[d]
 E11-2K,2 (20)	18 ± 5	w. ag.[e] (1.9% ± 1%)[f]
 E11-2K,3 (22)	13 ± 4	3.1 ± 2.1
 E11-3K,1 (28)	1.0 ± 0.5	not active
 E11-3K,2 (30)	3.7 ± 1.1	16 ± 11
 E11-3K,3 (32)	7.7 ± 1.0	13 ± 11

[a] Partial structure showing the side chain at the 11β-position of the E_2-analogue. [b] RBA of the indicated compound (compared to E_2 = 100%) to the ER in rat uterine cytosol. Values are ±SD. [c] K_i was determined by the inhibition of the stimulatory effect of 1 nM E_2 on alkaline phosphatase activity in the Ishikawa cells. [d] Weak mixed agonist/antagonist; weak activity that does not reach 50% of maximal and is reflected by low RBA in ER. [e] w.ag., weak agonist with low activity which does not approach 50% of maximal. [f] Values in parentheses are the relative stimulatory activity (RSA) in Ishikawa cells with respect to E_2.

Table 2. Estrogenic Properties of E_2-11β-Ethers

Compound[a]	RBA[b] (%) Rat uterine ER	RBA[b] (%) Human ERα-LBD	RBA[b] (%) Human ERβ-LBD	Inhibition[c] K_i nM
E11-0,4$_{ether}$ (40)	2.1 ± 0.2	7.5 ± 5.2	21 ± 14	not active.
E11-1,1$_{ether}$ (43)	20 ± 5	114 ± 45	240 ± 130	Agonist (125% ± 32%)[d]
E11-1,2$_{ether}$ (45)	28 ± 9	70 ± 32	73 ± 19	ag. / ant.[e]
E11-1,2F$_{ether}$ (54)	10 ± 6	27 ± 6	35 ± 10	w. ag. / ant.[f]
E11-1,3$_{ether}$ (47)	43 ± 5	146 ± 64	125 ± 98	0.3 ± 0.2
E11-1,4$_{ether}$ (49)	44 ± 10	102 ± 22	268 ± 112	0.1 ± 0.04
E11-2,1$_{ether}$ (56)	25 ± 13	67 ± 33	90 ± 35	ag. / ant.[e]
E11-2,2$_{ether}$ (58)	31 ± 16	49 ± 18	40 ± 17	0.3 ± 0.1
E11-2,2F$_{ether}$ (66)	16 ± 5	112 ± 37	34 ± 6	55 ± 42
E11-2,IPr$_{ether}$ (62)	15 ± 8	45 ± 18	32 ± 17	0.4 ± 0.3
E11-2,tBu$_{ether}$ (64)	2.3 ± 2.3	5.0 ± 2.0	2.0 ± 0.8	20 ± 13
E11-2,3$_{ether}$ (60)	65 ± 18	54 ± 17	88 ± 17	0.3 ± 0.2
E11-3,1$_{ether}$ (69)	34 ± 5	159 ± 38	111 ± 23	0.3 ± 0.2
E11-3,2$_{ether}$ (71)	48 ± 10	87 ± 21	75 ± 16	0.3 ± 0.2
E11-3,2F$_{ether}$ (78)	47 ± 15	96 ± 41	140 ± 47	1.1 ± 0.5
E11-3,IPr$_{ether}$ (74)	114 ± 25	220 ± 62	93 ± 18	0.2 ± 0.2
E11-3,tBu$_{ether}$ (76)	81 ± 18	128 ± 54	82 ± 16	0.09 ± 0.06

[a] Partial structure showing the side chain at the 11β-position of the E_2-analogue. [b] RBA (compared to E_2 = 100%) of the indicated E_2-11β-ether determined by inhibition of the binding of [^{3}H]E_2 to ER in rat uterine cytosol or lysates of *E. coli* in which the ligand binding domain (LBD) of human ERα and ERβ were separately expressed. [c] K_i was determined in Ishikawa cells as described in the legend to Table 1. [d] RSA with respect to E_2. [e] Mixed agonist/antagonist moderate activity that plateaus significantly below 100% of maximal activity (see Figure 2). [f] Weak mixed agonist/antagonist; weak activity that does not reach 50% of maximal activity and is reflected by low RBA in ER binding. Values are ±SD.

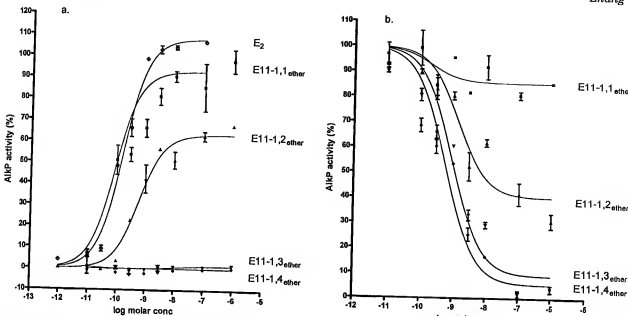


Figure 2. Estrogenic (a) and antiestrogenic (b) action of the E11-1 ethers (11β-side chain length of n = 3, E11-1,1$_{ether}$, through n = 6, E11-1,4$_{ether}$) on the expression of AlkP in Ishikawa cells. The cells were grown for 3 days with the indicated amounts of each E11-1 ether alone (a) or with 1 nM E_2 (b). In both panels 100% is the AlkP response normalized to 1 nM E_2 alone. E11-1,1$_{ether}$ is a full agonist (a), without significant effect on the action of E_2 (b). E11-1,2$_{ether}$ is defined as a mixed agonist/antagonist (ag/ant.) that alone stimulated AlkP (a) and inhibited E_2 action (b), but in neither case did the effect approach 100% activity. E11-1,3$_{ether}$ and E11-1,4$_{ether}$ are estrogen antagonists; inactive alone (a) but completely inhibit the stimulatory effect of E_2 (b). In both panels 100% is the AlkP response to 1 nM E_2. Error bars are SEM.

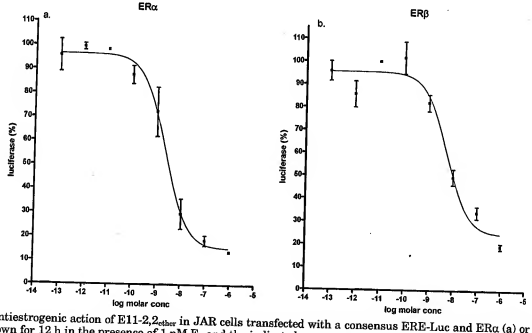


Figure 3. Antiestrogenic action of E11-2,2$_{ether}$ in JAR cells transfected with a consensus ERE-Luc and ERα (a) or ERβ (b). The cells were grown for 12 h in the presence of 1 nM E_2 and the indicated concentrations of E11-2,2$_{ether}$. In both panels, 100% is the luciferase response normalized to 1 nM E_2 alone. Error bars are SEM.

ovariectomized rats. However, these low doses produced striking effects on lowering plasma cholesterol. In a separate study the pure antiestrogen ICI 182,780[7] was coadministered with the higher dose (2) of E11-2,2$_{ether}$ (Figure 5, inset panel c). The antiestrogen had a small but statistically significant effect ($P < 0.001$) in blocking the agonist action of E11-2,2$_{ether}$ on plasma cholesterol.

Discussion

From the results of the ketone (Table 1) and ether analogue studies (Table 2) where $n \geq 5$, it is apparent that both the carbonyl group and the ester oxygen function composing the ester side chain are not required to be present together for antiestrogenic activity. In fact, those compounds lacking the carbonyl group, ethers, exhibit much higher antagonist activity than their corresponding esters.

For ethers of the same length (Table 2), the RBA for the ER appears to be uninfluenced by the position of the ether oxygen along the 11β-chain, with the exception of E11-0,4$_{ether}$, which is a poor ER ligand. Given the weak activity of this E_2-11β-ether, it is noteworthy that

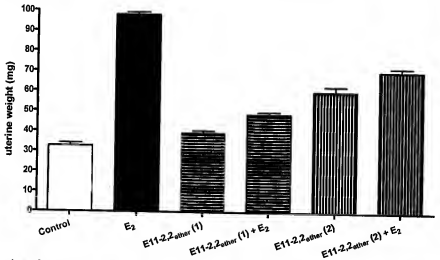


Figure 4. Antiestrogenic and estrogenic action of E11-2,2_{ether} on the uterus of the immature rat. Twenty-one day old Sprague–Dawley rats were injected with a total dose of 20 ng of E_2, 100 ng of E11-2,2_{ether} (1) or 1 μg of E11-2,2_{ether} (2) separately or together with 20 ng of E_2 (+ E_2) in 0.1 mL of sesame oil. The control group was injected with sesame oil alone. $n = 6$ animals/group. Error bars are SEM. Relevant statistical comparisons are provided in the text.

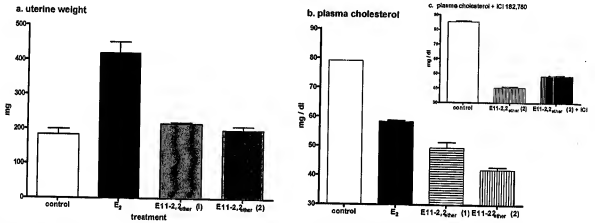


Figure 5. Tissue-specific effect of E11-2,2_{ether} on uterine weight (panel a) and liver (plasma cholesterol) (panel b) of the ovariectomized rat. Ovariectomized rats (~250 g) were treated sc daily for 7 days with 33 ng of E_2, 36 ng of E11-2,2_{ether} (1) or 180 ng E11-2,2_{ether} (2) in 0.1 mL of sesame oil. The control group was injected with sesame oil alone. $n = 6$ animals/group. (inset panel c) In a separate experiment ovariectomized animals were injected with 180 ng of E11-2,2_{ether} (2) or 180 ng of E11-2,2_{ether} (2) + ICI 182,780 in 0.1 mL of sesame oil. Error bars are SEM. Relevant statistical comparisons are provided in the text.

moving the ether oxygen anywhere else in the chain leads to compounds with good ER binding and, when $n \geq 5$, strong estrogen antagonism.

For steroidal esters of E_2 at 16α, estrogenic action is enhanced with fluorine at the terminus of the substituent.[1] We tested for a similar effect in compounds with fluorine incorporation at the 11β-ether side chain terminus, synthesizing E11-1,$2F_{ether}$, E11-2,$2F_{ether}$, and E11-3,$2F_{ether}$. However, the fluorine compounds E11-1,$2F_{ether}$ and E11-2,$2F_{ether}$ show, instead, a significant decrease in ER binding as compared to their unfluorinated counterparts E11-1,2_{ether} and E11-2,2_{ether}, respectively. In both fluorine compounds, the low RBAs were reflected in poor biological activity: E11-2,$2F_{ether}$ has a much decreased antiestrogenic action when compared to its unfluorinated counterpart E11-2,2_{ether}, and E11-1,$2F_{ether}$ is characterized as a weak agonist/antagonist (a compound that has a relatively large EC_{50} or K_i, respectively, and does not exceed 50% of maximal activity in each case) With E11-3,$2F_{ether}$, ER binding is unaffected, but estrogen antagonism is decreased by fluorine substitution as compared to E11-3,2_{ether}. Clearly, fluorine addition at the side chain terminus offers no advantage in the biological activity of these estradiol analogues.

We prepared a series of compounds analogous to E11-2,2_{ether} and E11-3,2_{ether} in which we incorporated branched methyl groups at the end of the 11β-ether side chain to investigate the possibility that a bulky side chain terminus might enhance antiestrogenic activity in compounds with good ER binding (Table 2). However, compounds having the side chain branch point too close to the steroid nucleus (E11-2,iPr_{ether}, E11-2,tBu_{ether}) exhibit lower binding to the ER. On the other hand, moving the branch one methylene unit out (E11-3,iPr_{ether}, E11-3,tBu_{ether}) enhances both ER binding and estrogen antagonism.

We have previously found in the 11β-ester series that E11-2,2 and E11-3,2 bound equally to the LBD of human ERα and human ERβ. ERα is the classical estrogen receptor found in most estrogen target organs, while ERβ is an ER isoform that is distributed in a more

restricted manner.[24] Both ERα and ERβ bind estrogens, but they have somewhat different affinity for particular estrogens. For example, C-16α-analogues of E_2 bind with a 20−90-fold ERα selectivity compared to ERβ.[1,26] However, most of the E_2-ethers in this study appear to bind about equally to each ER isoform, although in some cases there is modest selectivity (Table 2). Of note is the larger RBA observed for most of the ether analogues for both human ER LBDs compared to the ER in rat uterine cytosol. This is not unexpected, as it probably represents the temperature difference in the two assays. The uterine assay is run at 0−2 °C while the LBD assays are run at room temperature. It is well-known that this elevated temperature increases the RBA for 11β-analogues of E_2.[26−28] Furthermore, this difference in RBA could result from the source (rat and human) as well as a disparity between the intact ER and the ER receptor fragment (LBD). Regardless, the comparison of the potency of the analogues to each other in the various binding assays is the important point.

As we showed (Table 2), the 11β-ethers of E_2 have little binding selectivity for ERα or ERβ, and thus, it might be presumed that they would act similarly as antiestrogens with both ER isoforms. Indeed, we previously found that the antiestrogenic effect of the ester E11-2,2 is exhibited through both ERα and ERβ.[5] In the present study, we investigated the action of E11-2,2_{ether} in JAR cells that had been separately transfected with ERα and ERβ and a consensus ERE linked to a luciferase reporter gene. Like the ester E11-2,2, the ether E11-2,2_{ether} is an estrogen antagonist with both ER subtypes; it inhibits E_2-stimulated transcription of the reporter gene with both ERα and ERβ (Figure 3). The experiment shown in Figure 3 with E11-2,2_{ether} is representative of other experiments we performed with the other $n \geq 5$ 11β-ethers. Prior to these studies, it has been reported that other 11β-analogues of E_2, substituted with pure alkanes and alkenes from C_5 to C_9 in length show differential behavior; in cells transfected separately with the two estrogen receptor subtypes and a reporter gene, the alkanes and alkenes are ERβ antagonists, but to the contrary, they are ERα agonists.[29] This selective behavior between the two ER subtypes is not unprecedented. The *R,R*-enantiomer of tetrahydrochrysene (*R,R*-THC), which also has short alkyl side chains (two ethyl groups), is likewise an ERβ antagonist and an ERα agonist.[30] It is not clear what makes the E_2-11β-alkanes/alkenes ERα-agonists while all of the compounds described in this study are ERα-antagonists, but it seems that hydrogen binding to the ether oxygen must play a role. Since the E_2-11β substituted alkanes and alkenes are ERα agonists,[29] then E11-2,2_{ether}, which is a ERα antagonist, must be acting through a markedly different mechanism with ERα.

The antiestrogenic action of E11-2,2_{ether} was investigated further in vivo in the classical estrogen bioassay, determining its effect on the uterotrophic stimulation of E_2 in the immature rat. The rat uterus has a preponderance of ERα compared to ERβ,[31] and the uterotrophic response to estrogens has been shown to be ERα-ligand selective;[32] furthermore, the uterus of the ERα-knockout mouse does not respond to E_2.[33] These experiments confirm that E11-2,2_{ether} acts through ERα and demonstrate its mixed agonist/antiestrogenic action in the uterus in vivo (Figure 4). As might be expected, E11-2,2_{ether} was considerably more potent in this assay than the labile ester E11-2,2.[5]

While the SERM raloxifene exhibits an antiestrogenic effects in specific tissues, such as the uterus, in other tissues, however, including the liver, it is estrogenic.[34] This is manifested by a decrease in plasma cholesterol, a well-known estrogenic action on the liver, which in the rat produces an increase in hepatic LDL receptors, resulting in a concomitant clearance of LDL and HDL cholesterol.[6] As with the antiestrogenic action on the uterus (above), the effect of E11-2,2_{ether} on plasma cholesterol is much more potent than that which we previously found for the labile ester E11-2,2.[5] This follows from the fact that the ethers such as E11-2,2_{ether}, in contrast to the esters, are not susceptible to esterase hydrolysis. It is noteworthy that E11-2,2_{ether} produces an agonist effect on plasma cholesterol at a dose that has no effect on the uterus (Figure 5). As would be expected, the pure antiestrogen ICI 187,780 inhibited the agonist action of E11-2,2_{ether} on plasma cholesterol (Figure 5, inset panel c). While the agonist activity of E11-2,2_{ether} was significantly blocked ($P < 0.001$), the effect was comparatively small as the pure antagonist ICI 187,780 increased the cholesterol levels of the E11-2,2_{ether}-treated rats only slightly. This is not unexpected, as it has been observed previously that the antiestrogen is remarkably less potent in inhibiting estrogenic stimulation of the liver (plasma cholesterol) compared to the uterus.[35]

Like the ester E11-2,2, these studies demonstrate that E11-2,2_{ether} is a SERM: it is antiestrogenic in the Ishikawa cell and in JAR cells transfected with ERα or ERβ, in vivo in the uterus it is an antagonist with weak agonist activity, and in the liver it is estrogen agonist. These nonpolar short 11β-side chain analogues of E_2 are different from the classical antiestrogens and SERMs that uniformly contain a long and polar or charged side chain.[36] This side chain is essential for the antiestrogenic action, as it interferes with the agonist conformation of helix 12 in the ER and consequently binding of required coactivators.[8,9,11] This suggests the possibility that these ethers act on ER by a different mechanism (perhaps similar to the mechanism by which the short-chain ERβ-antagonist THC acts on ERβ[37]), and thus, they could be unique therapeutic agents. The increased antiestrogenic action of the analogues with bulky side chains, E11-3,iPr_{ether} and E11-3,tBu_{ether}, indicates that other sterically constrained analogues might have even greater activity, and studies investigating this possibility are underway in this laboratory.

Experimental Section

General Methods. ^1H NMR spectra were recorded with a Bruker Avance 400 or Avance 500 spectrometer, and chemical shifts are reported relative to residual $CHCl_3$ (7.27 ppm) or acetone-d_6 (2.05 ppm). Purification by flash chromatography was performed according to the procedure of Still[38] using 230−400 mesh silica gel (EM Science, Darmstadt Germany). High-resolution mass spectra were obtained by electrospray ionization on a Micromass Q-Tof spectrometer by Dr. Walter J. McMurray at the Yale University Comprehensive Cancer Center/WM Keck Biotechnology Resource Center using NaI as an internal standard. The computer program Prism was purchased from GraphPad Software, Inc. (San Diego, CA). The cell culture reagents were obtained form Gibco-BRL (Grand

Island, NH). The synthesis of compounds **1**, **4**, **12**, **18**, **41**, and **67** were previously described.[3] Unless otherwise noted, solvents (analytical or HPLC) and reagents were used as supplied, and all reactions were carried out under nitrogen. ICI 182,780 was obtained from Tocris Cookson Inc (Ellisville, MO).

Chromatographic Systems. Thin-layer chromatography (TLC) was performed using Merck silica gel plates (F254) (EM Science) and visualized using phosphomolybdic acid or UV illumination. TLC systems: T-1, EtOAc/hexanes (2:1); T-2, hexanes/EtOAc (6:1); T-3, hexanes/EtOAc (2:1); T-4, hexanes/EtOAc (4:1); T-5, hexanes/EtOAc (1:1); T-6, hexanes/EtOAc (3:1); T-7, hexanes/EtOAc (1:3). Analytical high-performance liquid chromatography (HPLC) was performed on a Beckman System Gold HPLC system (Beckman Coulter, Inc. Fullerton, CA) consisting of a model 126 solvent module and a model 168 diode array detector at 210 and 280 nm using the following columns and systems. With an Ultrasphere ODS column (5 μm, 10 mm × 25 cm, Altex Scientific Operations Co.), the following solvent systems at 3 mL/min were used, unless otherwise noted: H-1, CH_3CN/H_2O (1:1); H-2, CH_3CN/H_2O (65:35); H-3, CH_3CN/H_2O (55:45); H-4, CH_3CN/H_2O (45:55); H-5, CH_3CN/H_2O (40:60); H-6, $CH_3CN/H_2O/HOAc$ (42:57.884:0.116); H-7, $CH_3CN/H_2O/HOAc$ (40:59.88:0.12); H-8, CH_3CN/H_2O (60:40); H-9, CH_3OH/H_2O (60:40), 2 mL/min; H-10, CH_3OH/H_2O (70:30), 1.5 mL/min; H-11, CH_3OH/H_2O (75:25), 1.2 mL/min. With a LiChrospher 100 Diol column (5 μm, 4.6 mm × 25 cm, EM Science) the following solvent systems at 1 mL/min were used: H-12, CH_2Cl_2; H-13, CH_2Cl_2/iPrOH (92:8); H-14, CH_2Cl_2/iPrOH (90:10); H-15, CH_2Cl_2/iPrOH (99:1); H-16, CH_2Cl_2/isooctane (90:10); H-17, CH_2Cl_2/iPrOH (97:3); H-18, CH_2Cl_2/iPrOH (98:2). With a Protein I-60 column (7.8 mm × 30 cm, Waters Co.) the following solvent system at 3 mL/min was used: H-19, CH_2Cl_2 at 2 mL/min. With a Microsorb-MV C18 column (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) the following solvent systems were used at 1 mL/min: H-20, CH_3CN/H_2O (1:1); H-21, CH_3CN/H_2O (40:60).

Isopropyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)acetate (2, E11-2,iPr). A solution of 10.0 mg (0.030 mmol) of **1** and 100 μL (163 mg, 1.37 mmol) of $SOCl_2$ in 2-propanol (1 mL) was stirred and heated at 60 °C in a sealed vial for 24 h. The reaction mixture was poured into saturated aqueous $NaHCO_3$ (50 mL) and extracted with EtOAc (3×, 50 mL). Combined organic extracts were washed with H_2O (20 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by HPLC using system H-1 gave 5.6 mg (49%) of **2** as a white solid. Data for **2**: TLC, T-1, R_f 0.63; ^1H NMR (400 MHz, $CDCl_3$) δ 0.91 (s, 3H, H-18), 1.17 (d, 3H, J = 6.2 Hz, OCH(CH_3)$_2$), 1.22 (d, 3H, J = 6.2 Hz, OCH(CH_3)$_2$), 3.70 (m, 1H, H-17α), 4.98 (septet, 1H, J = 6.2 Hz, $OCH(CH_3)_2$), 6.53 (d, 1H, J = 2.2 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 7.08 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES^+) calcd for $C_{23}H_{32}O_4$-Na (M + Na^+) *m/e* 395.2198, found *m/e* 395.2197; HPLC system H-20, t_R = 10.2 min, and system H-12, t_R = 12.5 min, >99% pure.

Propyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)-acetate (3, E11-2,3). Compound **3** was prepared by esterification of **1** (8.5 mg, 0.026 mmol) with *n*-propanol as described for **2**. Purification by HPLC using system H-1 gave 6.3 mg (66%) of **3** as a white solid. Data for **3**: ^1H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.5 Hz, $-CH_2CH_3$), 0.93 (s, 3H, H-18), 3.73 (m, 1H, H-17α), 4.00 (m, 2H, $-OCH_2-$), 6.55 (d, 1H, J = 2.5 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 2.5 Hz, H-2), 7.08 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES^+) calcd for $C_{23}H_{32}O_4Na$ (M + Na^+) *m/e* 395.2198, found *m/e* 395.2197; HPLC system H-20, t_R = 10.6 min, and system H-12, t_R = 12.9 min, >99% pure.

2,2-Dimethylpropyl (3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)acetate (5). A solution of 20.0 mg (0.039 mmol) of **4**, 15 mg (0.170 mmol) of neopentyl alcohol, 1 mg (0.008 mmol) of DMAP, and 8 mg (0.04 mmol) of 1,3-dicyclohexylcarbodiimide in CH_2Cl_2 (1 mL) was stirred and heated at 50 °C for 24 h. The reaction mixture was cooled to room temperature, filtered, and washed through with CH_2Cl_2. The filtrate was dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using 7:1 hexanes/EtOAc as eluent gave 14 mg (61%) of **5** as a white solid.

2,2-Dimethylpropyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)acetate (6, E11-2,diMePr). At 0 °C, BCl_3 (0.4 mL, 1.0 M solution in CH_2Cl_2) was added to a solution of compound **5** (10 mg, 0.017 mmol) in CH_2Cl_2 (1.5 mL). The reaction was stirred at 0 °C for 30 min, quenched with H_2O (10 mL), and extracted with EtOAc (3×, 20 mL). Combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification by HPLC using system H-2 (t_R = 7.5 min) gave 3 mg (46%) of **6** as a white solid. Data for **6**: ^1H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 9H, $-C(CH_3)_3$), 0.96 (s, 3H, H-18), 3.74 (t, 2H, J = 7.4 Hz, H-17α), 3.73 & 3.79 (AB quartet, 2H, J_{AB} = 10.6 Hz, $-OCH_2-$), 6.57 (d, 1H, J = 2.7 Hz, H-4), 6.66 (dd, 1H, J = 8.3, 2.7 Hz, H-2), 7.10 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES^+) calcd for $C_{28}H_{36}O_4Na$ (M + Na^+) *m/e* 423.2511, found *m/e* 423.2525; HPLC system H-20, t_R = 16.4 min, and system H-12, t_R = 10.9 min, >99% pure.

Ethyl (3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)-acetate (7). Compound **7** was prepared from **4** (140 mg, 0.274 mmol) and $SOCl_2$ (700 μL, 9.5 mmol) in EtOH (25 mL) as described for **2**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 6:1 hexanes/EtOAc as eluent gave 147 mg (100%) of **7**: TLC, T-2, R_f 0.68.

***O*-Ethyl (3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)thioacetate (8).** A solution of 10 mg (0.018 mmol) of **7** and 20 mg (0.049 mmol) of Lawesson's reagent[13] in o-xylene (1 mL) was stirred and heated at 140 °C in a sealed vial for 24 h. The reaction mixture was allowed to cool to room temperature and subjected to flash chromatography on a 2 × 17 cm column of silica gel using 25:1 hexanes/EtOAc as eluent, giving 3 mg (28%) of **8** (TLC system T-2, R_f 0.52) and 5.6 mg of recovered 7 (TLC system T-2, R_f 0.34). Data for **8**: ^1HNMR (400 MHz, $CDCl_3$) δ 1.06 (s, 3H, H-18), 1.40 (t, 3H, J = 7.0 Hz, $-CH_2CH_3$), 3.50 (t, 1H, J = 7.9 Hz, H-17α), 4.47–4.57 (m, 2H, $-OCH_2-CH_3$), 4.57 (s, 2H, OBn), 5.04 (s, 2H, OBn), 6.71 (d, 1H, J = 2.5 Hz, H-4), 6.82 (dd, 1H, J = 8.7, 2.5 Hz, H-2), 7.22 (d, 1H, J = 8.7 Hz, H-1), 7.30–7.46 (m, 10H, Ar–H).

***O*-Ethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)-thioacetate (9, E11-2S,2).** At 0 °C, BCl_3 (1.0 mL, 1.0 M solution in CH_2Cl_2) was added to a solution of compound **8** (25 mg, 0.045 mmol) in CH_2Cl_2 (1.5 mL). The reaction was stirred at 0 °C for 30 min, quenched with saturated aqueous $NaHCO_3$ (10 mL), and extracted with EtOAc (3×, 20 mL). Combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification by HPLC using system H-1 gave 5 mg (31%) of **9** as a white solid. Data for **9**: TLC, T-3, R_f 0.4; ^1H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.40 (t, 3H, J = 7.1 Hz, $-OCH_2CH_3$), 3.73 (m,1H, H-17α), 4.49–4.55 (m, 2H, $-OCH_2CH_3$), 6.56 (d, 1H, J = 2.7 Hz, H-4), 6.67 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.17 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES^+) calcd for $C_{22}H_{30}O_3SNa$ (M + Na^+) *m/e* 397.1813, found *m/e* 397.1813; HPLC system H-20, t_R = 14.6 min, and system H-12, t_R = 9.4 min, >99% pure.

***N*-Ethyl(3,17β-dibenzyloxyestra-1,3,5(10)-trien-11β-yl)-acetamide (10).** The synthesis of **10** is based on the literature procedure.[14] A mixture of 32 mg (0.063 mmol) of **4**, 24 mg (0.15 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, 13 mg (0.11 mmol) of DMAP, and 29 mg (51 μL, 0.64 mmol of a 70% aqueous solution) of ethylamine in CH_2Cl_2 (2.5 mL) was stirred at room temperature for 24 h. The reaction mixture was poured into CH_2Cl_2 (50 mL) and washed with saturated aqueous NH_4Cl (2×, 50 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of flash silica gel using 2:1 hexanes/EtOAc as eluent gave 12 mg (36%) of **10**. Data for **10**: TLC, T-5, R_f 0.66; ^1H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 3H, H-18), 1.10 (t, 3H, J = 7.1 Hz, $-CH_2CH_3$), 2.29 (d, 1H, J = 13.6 Hz, H-12β), 2.60 (dd, 1H, J = 10.7, 5.2 Hz, H-9), 2.72–2.86 (m, 2H, H-6), 3.16–3.36 (m, 3H, H-11, $-CH_2-CH_3$), 3.51 (t, 1H, J = 8.9 Hz, H-17α), 4.56 & 4.59 (AB quartet, 2H, J_{AB} = 12.3 Hz, OBn), 5.03 (s, 2H, OBn), 5.17 (br s, 1H,

NH), 6.70 (d, 1H, J = 2.6 Hz, H-4), 6.80 (dd, 1H, J = 8.9, 2.6 Hz, H-2), 7.19 (d, 1H, J = 8.9 Hz, H-1), 7.30–7.45 (m, 10H, Ar–H).

***N*-Ethyl(3,17β-dihydroxyestra-1,3,5(10)-trien-11β-yl)-acetamide (11, E11-2,$_{amide}$).** Compound **11** was prepared from **10** (12 mg, 0.022 mmol) as described for **6**. Purification by HPLC using system H-5 gave 2.4 mg (30%) of **11** as a white solid. Data for **11**: TLC, T-7, R_f 0.125; ^{1}H NMR (400 MHz, acetone-d_6) δ 0.93 (s, 3H, H-18), 1.04 (t, 3H, J = 7.3 Hz, $-CH_2CH_3$), 2.53 (dd, 1H, J = 10.8, 4.8 Hz, H-9), 2.63–2.79 (m, 2H, H-6), 3.08–3.22 (m, 3H, H-11, $-CH_2CH_3$), 3.65 (t, 1H, J = 8.2 Hz, H-17α), 6.52 (d, 1H, J = 2.5 Hz, H-4), 6.62 (dd, 1H, J = 8.3, 2.5 Hz, H-2), 6.93 (br s, 1H, NH), 7.06 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{31}NO_3Na$ (M + Na$^+$) m/e 380.2202, found m/e 380.2202; HPLC system H-21, t_R = 7.9 min, and system H-13, t_R = 7.25 min, >99% pure.

***N*-Ethyl-3-(3,17β-dibenzyloxyestra-1,3,5(10)-trien-11β-yl)propionamide (13).** Compound **13** was prepared from **12** (30 mg, 0.057 mmol) as described for **10**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1.5:1 hexanes/EtOAc as eluent gave 18 mg (57%) of **13**. Data for **13**: TLC, T-5, R_f 0.58; ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.03 (s, 3H, H-18), 1.14 (t, 3H, J = 7.2 Hz, $-CH_2CH_3$), 2.28 (d, 1H, J = 13.6 Hz, H-12β), 2.58 (dd, 1H, J = 10.6, 4.3 Hz, H-9), 2.69–2.86 (m, 2H, $-CH_2CH_3$), 3.24–3.31 (m, 2H, $-CH_2CH_3$), 3.49 (t, 1H, J = 8.0 Hz, H-17α), 4.57 & 4.61 (AB quartet, 2H, J_{AB} = 12.2 Hz, OBn), 5.04 (s, 2H, OBn), 5.31 (br s, 1H, NH), 6.69 (d, 1H, J = 2.5 Hz, H-4), 6.81 (dd, 1H, J = 8.5, 2.5 Hz, H-2), 7.11 (d, 1H, J = 8.5 Hz, H-1), 7.30–7.46 (m, 10H, Ar–H).

***N*-Ethyl-3-(3,17β-dihydroxyestra-1,3,5(10)-trien-11β-yl)-propionamide (14, E11-3,$_{amide}$).** Compound **14** was prepared from **13** (9 mg, 0.016 mmol) as described for **6**. Purification by HPLC using system H-5 gave 3 mg (50%) of **14** as a white solid. Data for **14**: ^{1}H NMR (400 MHz, acetone-d_6) δ 0.93 (s, 3H, H-18), 1.05 (t, 3H, J = 7.3 Hz, $-CH_2CH_3$), 3.13–3.20 (m, 2H, $-CH_2CH_3$), 3.64 (t, 1H, J = 8.3 Hz, H-17α), 6.52 (dd, 1H, J = 2.6 Hz, H-4), 6.62 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 6.95 (br s, 1H, NH), 7.05 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{33}NO_3Na$ (M + Na$^+$) m/e 394.2358, found m/e 394.2356; HPLC system H-20, t_R = 6.3 min, and system H-14, t_R = 5.8 min, >99% pure.

Ethyl (3,17β-dibenzyloxyestra-1,3,5(10)-trien-11β-yl)-propionate (15). Compound **15** was prepared from **12** (110 mg, 0.210 mmol) and SOCl$_2$ (0.2 mL, 2.74 mmol) in EtOH (3 mL) at 45 °C as described for **2**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 7:1 hexanes/EtOAc as eluent gave 85 mg (73%) of **15**.

***N*-Methyl-3-(3,17β-dibenzyloxyestra-1,3,5(10)-trien-11β-yl)propionamide (16).** The synthesis of **16** is based on the literature procedure.[15,16] A solution of 20 mg (0.036 mmol) of **15** and 1.3 mg (0.026 mmol) of NaCN in 33% ethanolic CH$_3$-NH$_2$ (2 mL) was stirred and heated at 60 °C for 20 h. The solvent was evaporated under a N_2 stream and the residue was purified by flash chromatography using 2:1 hexanes/EtOAc as eluent to give 15 mg (77%) of **16**. Data for **16**: TLC, T-3, R_f 0.27; ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.03 (s, 3H, H-18), 2.69–2.86 (m, 2H, H-6), 2.78 (d, 3H, J = 4.9 Hz, NCH$_3$), 3.49 (t, 1H, J = 8.1 Hz, H-17α), 4.57 & 4.60 (AB quartet, 2H, J_{AB} = 12.3 Hz, OBn), 5.04 (s, 2H, OBn), 5.33 (br s, 1H, NH), 6.70 (d, 1H, J = 2.7 Hz, H-4), 6.81 (dd, 1H, J = 8.6, 2.7 Hz, H-2), 7.10 (d, 1H, J = 8.6 Hz, H-1), 7.30–7.46 (m, 10H, Ar–H).

***N*-Methyl-3-(3,17β-dihydroxyestra-1,3,5(10)-trien-11β-yl)propionamide (17, E11-3,$_{amide}$).** Compound **17** was prepared from **16** (15 mg, 0.028 mmol) as described for **6**. Purification by HPLC using system H-5 gave 3.5 mg (35%) of **17** as a white solid. Data for **17**: ^{1}H NMR (400 MHz, acetone-d_6) δ 0.93 (s, 3H, H-18), 2.65 (d, 3H, J = 4.9 Hz, NCH$_3$), 3.64 (dd, 1H, J = 8.8, 7.4 Hz, H-17α), 6.52 (d, 1H, J = 2.3 Hz, H-4), 6.62 (dd, 1H, J = 8.3, 2.3 Hz, H-2), 6.93 (br s, 1H, NH), 7.05 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{31}NO_3Na$ (M + Na$^+$) m/e 380.2202, found m/e 380.2203; HPLC system H-21, t_R = 8.5 min, and system H-13, t_R = 10 min, >99% pure.

1-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)butan-2-one (19). The synthesis of **19** is based on the literature procedure.[17] To a solution of 30 mg (0.061 mmol) of **18** in 1 mL of Et$_2$O was added 60 μL (0.18 mmol) of a 3 M solution of ethylmagnesium bromide in Et$_2$O and reaction was stirred at room temperature for 24 h. To the reaction mixture was added Et$_2$O (1 mL) and 1 M HCl (1 mL) and stirring was continued at room temperature for 2 h. The reaction was poured into H$_2$O (50 mL) and extracted with EtOAc (3×, 30 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 6:1 hexanes/EtOAc as eluent gave 23 mg (72%) of **19**. TLC, T-4, R_f 0.62.

1-(3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)butan-2-one (20, E11-2K,2). Compound **20** was prepared from **19** (20 mg, 0.038 mmol) as described for **6**. Purification by HPLC using system H-1 gave 9.5 mg (72%) of **20** as a white solid. Data for **20**: ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.91 (s, 3H, H-18), 0.98 (t, 3H, J = 7.3 Hz, $-CH_2CH_3$), 2.70–2.86 (m, 2H, H-6), 3.21–3.24 (m, 1H, H-11), 3.74 (dd, 1H, J = 9.0, 7.4 Hz, H-17α), 6.57 (d, 1H, J = 2.6 Hz, H-4), 6.61 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 6.97 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{30}O_3Na$ (M + Na$^+$) m/e 365.2093, found m/e 365.2082; HPLC system H-20, t_R = 8.1 min, and system H-19, t_R = 9.4 min, >99% pure.

1-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)pentan-2-one (21). Compound **21** was prepared from **18** (420 mg, 0.854 mmol) and propylmagnesium chloride (2.8 mL of a 2.0 M solution in Et$_2$O, 5.6 mmol) as described for **19**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 6:1 hexanes/EtOAc as eluent gave 400 mg (87%) of **21**. Data for **21**: TLC, T-4, R_f 0.56; ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.87 (t, 3H, J = 7.3 Hz, $-CH_2CH_3$), 0.99 (s, 3H, H-18), 2.73–2.85 (m, 2H, H-6), 3.19–3.25 (m, 1H, H-11), 3.49 (t, 1H, J = 7.9 Hz, H-17α), 4.56 (s, 2H, OBn), 5.03 (s, 2H, OBn), 6.71 (d, 1H, J = 2.5 Hz, H-4), 6.75 (dd, 1H, J = 8.6, 2.5 Hz, H-2), 7.00 (d, 1H, J = 8.6 Hz, H-1), 7.29–7.45 (m, 10H, Ar–H).

1-(3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)pentan-2-one (22, E11-2K,3). Compound **22** was prepared from **21** (30 mg, 0.056 mmol) as described for **6**. Purification by HPLC using system H-3 gave 10 mg (50%) of **22**. Data for **22**: ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.86 (t, 3H, J = 7.4 Hz, $-CH_2CH_3$), 0.91 (s, 3H, H-18), 2.70–2.85 (m, 2H, H-6), 3.19–3.24 (m, 1H, H-11), 3.74 (t, J = 6.6 Hz, H-17α), 6.57 (d, 1H, J = 2.5 Hz, H-4), 6.60 (dd, 1H, J = 8.5, 2.5 Hz, H-2), 6.97 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{23}H_{32}O_3Na$ m/e 379.2249, found m/e 379.2241; HPLC system H-20, t_R = 11.2 min, and system H-19, t_R = 8.4 min, > 99% pure.

2-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)ethanol (24). A solution of 605 mg (1.26 mmol) of **23**[18] and 43.0 mg (1.97 mmol) of LiBH$_4$ in a 1 M solution of catecholborane in THF was stirred at room temperature for 21 h. The reaction mixture was poured into a 0 °C solution of NaOH (0.57 g), EtOH (6 mL), H_2O_2 (6 mL), and H$_2$O (1.4 mL) and allowed to stir at room temperature for 6 h. The reaction mixture was then poured into H$_2$O (70 mL) and extracted with EtOAc (3×, 70 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a slightly yellow oil. Purification by flash chromatography on a 3 × 21 cm column of silica gel using 2:1 hexanes/EtOAc as eluent gave a colorless oil which was dissolved in EtOAc (100 mL) and washed with 15% aqueous NaOH (50 mL) and H$_2$O (until the aqueous phase was colorless), giving 0.5 g (80%) of **24**. Data for **24**: TLC, T-6, R_f 0.18; ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.02 (s, 3H, H-18), 2.25 (dd, 1H, J = 13.8, 1.5 Hz, H-12β), 2.54–2.86 (m, 4H, H-6, 9, 11), 3.48 (dd, 1H, J = 8.7, 7.3 Hz, H-17α), 3.64–3.80 (m, 2H, $-CH_2O$), 4.57 & 4.58 (AB quartet, 2H, J_{AB} = 12.3 Hz, OBn), 5.03 (s, 2H, OBn), 6.70 (d, 1H, J = 2.8 Hz, H-4), 6.80 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.10 (d, 1H, J = 8.5 Hz, H-1), 7.29–7.49 (m, 10H, Ar–H).

2-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)ethyl Toluenesulfone (25). A solution of 68 mg (0.14 mmol) of **24** and 390 mg (2.01 mmol) of pTsCl in pyridine (5 mL) was allowed to stand at 4 °C for 6 days. Reaction was poured into H$_2$O (70 mL) and extracted with CH_2Cl_2 (3×, 70 mL). Combined organic extracts were dried over Na_2SO_4 and concen-

trated in vacuo. Purification by flash chromatography on a 2 × 21 cm column of silica gel using 4:1 hexanes/EtOAc as eluent gave 60 mg (68%) of **25**. Data for **25**: TLC, T-6, R_f 0.78; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (s, 3H, H-18), 2.41 (s, 3H, $-CH_3$), 3.45 (t, 1H, J = 7.9 Hz, H-17α), 4.08−4.17 (m, 2H, $-CH_2O$), 4.54 (s, 2H OBn), 5.04 (s, 2H, OBn), 6.69 (d, 1H, J = 2.6 Hz, H-4), 6.77 (dd, 1H, J = 8.5, 2.6 Hz, H-2), 7.00 (d, 1H, J = 8.5 Hz, H-1), 7.30−7.47 (m, 12H, Ar−H), 7.79 (d, 2H, J = 8.1 Hz, Ar−H).

3-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)propanenitrile (26). A solution of 220 mg (0.34 mmol) of **25** and 100 mg (2.0 mmol) of NaCN in DMSO (8 mL) was stirred and heated at 90 °C for 1.5 h. The reaction was allowed to cool to room temperature, poured into saturated aqueous NH_4Cl (50 mL), and extracted with CH_2Cl_2 (2×, 50 mL). Combined organic extracts were washed with H_2O (20 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 5:1 hexanes/EtOAc gave 150 mg (88%) of **26**. Data for **26**: TLC, T-4, R_f = 0.51; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.99 (s, 3H, H-18), 3.51 (t, 1H, J = 8.0 Hz, H-17α), 4.63 & 4.56 (AB quartet, 2H, J_{AB} = 12.4 Hz, OBn), 5.06 (s, 2H, OBn), 6.73 (d, 1H, J = 2.5 Hz, H-4), 6.85 (dd, 1H, J = 8.6, 2.5 Hz, H-2), 7.12 (d, 1H, J = 8.6 Hz, H-1), 7.30−7.48 (m, 10H, Ar−H).

4-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)butan-2-one (27). Compound **27** was prepared from **26** (27 mg, 0.053 mmol) and methylmagnesium bromide (120 μL of a 3.0 M solution in Et_2O, 0.36 mmol) in Et_2O (1.5 mL) as described for **19**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 8:1 hexanes/EtOAc gave 14 mg (50%) of **27**.

4-(3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)butan-2-one (28, E11-3K,1). Compound **28** was prepared from **27** (13 mg, 0.25 mmol) as described for **6**. Purification by HPLC using system H-1 gave 2 mg (23%) of **28** as a white solid. Data for **28**: TLC, T-5, R_f 0.35; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 2.12 (s, 3H, $-CH_3$), 2.67−2.82 (m, 2H, H-6), 3.73 (dd, 1H, J = 8.9, 7.3 Hz, H-17α), 6.55 (d, 1H, J = 2.5 Hz, H-4), 6.65 (dd, 1H, J = 8.5, 2.5 Hz, H-2), 7.06 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{30}O_3Na$ (M + Na$^+$) *m/e* 365.2093, found *m/e* 365.2096; HPLC system H-9, t_R = 26 min, and system H-15, t_R = 11.0 min, >99% pure.

1-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)pentan-3-one (29). Compound **29** was prepared from **26** (32 mg, 0.063 mmol) and ethylmagnesium bromide (130 μL of a 3 M solution in Et_2O, 0.39 mmol) as described for **19**. Purification by flash chromatography using 8:1 hexanes/EtOAc as eluent gave 22 mg (65%) of **29**: TLC, T-4, R_f 0.61.

1-(3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)pentan-3-one (30, E11-3K2). Compound **30** was prepared from **29** (20 mg, 0.037 mmol) as described for **6**. Purification by HPLC using system H-1 gave 3.7 mg (28%) of **30** as a white solid. Data for **30**: TLC, T-5, R_f 0.45; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 3H, H-18), 1.04 (t, 3H, J = 7.3 Hz, $-CH_3$), 2.67−2.85 (m, 2H, H-6), 3.73 (t, 1H, J = 8.0 Hz, H-17α), 6.55 (d, 1H, J = 2.7 Hz, H-4), 6.66 (dd, 1H, J = 8.4, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{23}H_{32}O_3Na$ (M + Na$^+$) *m/e* 379.2249, found *m/e* 379.2260; HPLC system H-10, t_R = 43 min, and system H-15, t_R = 8.2 min, >99% pure.

1-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)hexan-3-one (31). Compound **31** was prepared from **26** (30 mg, 0.059 mmol) and propylmagnesium chloride (200 μL of a 2 M solution in Et_2O, 0.4 mmol) as described for **19**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 10:1 hexanes/EtOAc as eluent gave 24 mg (73%) of **31**. Data for **31**: TLC, T-4, R_f 0.71; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.4 Hz, $-CH_3$), 1.02 (s, 3H, H-18), 2.70−2.87 (m, 2H, H-6), 3.49 (t, 1H, J = 7.9 Hz, H-17α), 4.58 & 4.61 (AB quartet, 2H, J_{AB} = 12.3 Hz, OBn), 5.04 (s, 2H, OBn), 6.70 (d, 1H, J = 2.4 Hz, H-4), 6.81 (dd, 1H, J = 8.6, 2.4 Hz, H-2), 7.11 (d, 1H, J = 8.6 Hz, H-1), 7.29−7.47 (m, 10H, Ar−H).

1-(3,17β-Dihydroxyestra-1,3,5(10)-trien-11β-yl)hexan-3-one (32, E11-3K,3). Compound **32** was prepared from **31** (20 mg, 0.36 mmol) as described for **6**. Purification by HPLC using system H-1 gave 3.6 mg (27%) of **32** as a white solid. Data for **32**: TLC, T-1, R_f 0.55; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (t, 3H, J = 7.6 Hz, $-CH_3$), 0.93 (s, 3H, H-18), 2.67−2.85 (m, 2H, H-6), 3.73 (dd, 1H, J = 8.8, 7.4 Hz, H-17α), 6.55 (d, 1H, J = 2.7 Hz, H-4), 6.66 (dd, 1H, J = 8.4, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{24}H_{34}O_3Na$ (M + Na$^+$) *m/e* 393.2406, found *m/e* 393.2402; HPLC system H-11, t_R = 29 min, and system H-15, t_R = 8.2 min, >99% pure.

3,11β-Dihydroxyestra-1,3,5(10)-trien-17-one (34). A solution of 1.13 g (3.4 mmol) of 33[19] in 1% KOH−MeOH (25 mL) was stirred and heated at 50 °C for 1 h. The reaction mixture was poured into H_2O (200 mL) and adjusted to pH 2 with concentrated HCl. The resulting white solid was collected by vacuum filtration and used without further purification in the next step. Data for **34**: TLC, T-5, R_f 0.31; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17 (s, 3H, H-18), 2.81−2.96 (m, 2H, H-6), 4.79 (dd, 1H, J = 5.6, 2.4 Hz, H-11), 6.64 (d, 1H, J = 2.6 Hz, H-4), 6.70 (dd, 1H, J = 8.3, 2.6 Hz, H-2), 7.17 (d, 1H, J = 8.3 Hz, H-1).

3-Benzyloxy-11β-hydroxyestra-1,3,5(10)-trien-17-one (35). A solution of 2.74 g (19.8 mmol) of K_2CO_3 in $CHCl_3$ (60 mL) and MeOH (30 mL) was stirred and heated at 70 °C for 20 min. To this was added a solution of crude 34 in $CHCl_3$ (40 mL) and MeOH (20 mL) followed by 600 μL (5.06 mmol) of benzyl bromide. The reaction mixture was stirred at reflux for 6 h then allowed to stir at room temperature overnight. The reaction mixture was poured into H_2O (300 mL) and extracted with EtOAc (2×, 250 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 3 × 17 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 1.09 mg (77%, two steps) of **35**. Data for **35**: TLC, T-5, R_f 0.64; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17 (s, 3H, H-18), 2.84−2.98 (m, 2H, H-6), 4.79 (dd, 1H, J = 5.5, 2.6 Hz, H-11), 5.06 (s, 2H, OBn), 6.78 (d, 1H, J = 2.7 Hz, H-4), 6.85 (dd, 1H, J = 8.6, 2.7 Hz, H-2), 7.21 (d, 1H, J = 8.6 Hz, H-1), 7.32−7.45 (m, 5H, Ar−H).

3-Benzyloxy-17,17-ethylenedioxyestra-1,3,5(10)-trien-11β-ol (36). A solution of 990 mg (2.63 mmol) of **35**, ethylene glycol (17.4 mL), and pTsOH (100 mg) in benzene (150 mL) was stirred and heated at reflux for 4 h in a 250 mL flask equipped with a reflux condenser and a Dean−Stark trap. The trap was emptied and 4Å sieves were added to it and heating was continued for additional 1 h. The reaction mixture was allowed to cool to room temperature, poured into saturated aqueous $NaHCO_3$ (200 mL), and extracted with EtOAc (2×, 200 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 3 × 17 cm column of silica gel using 2:1 hexanes/EtOAc as eluent gave 985 mg (89%) of **36**.

3-Benzyloxy-11β-butoxy-17,17-ethylenedioxyestra-1,3,5-(10)-triene (37). A suspension of 47 mg (16 mg, 0.41 mmol) of a 35% dispersion of KH in mineral oil was washed in a 10 mL pear flask with hexanes (3 mL) and resuspended in anhydrous toluene (1 mL). To this was added a solution of 115 mg (0.273 mmol) of **36** in 2 mL of toluene, and the reaction mixture was stirred and heated at 80 °C for 30 min. To this was added a solution of 312 mg (1.36 mmol) of butyl toluenesulfonate[20] in toluene (1 mL) and stirring was continued at 80 °C for 20 h. The reaction mixture was allowed to cool to room temperature, poured into saturated aqueous NH$_4$Cl (200 mL), and extracted with CH_2Cl_2 (2×, 150 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 21 cm column of silica gel using 5:1 hexanes/EtOAc as eluent gave 264 mg of product containing material which was used in the next step without further purification. Integration of the ^{1}H NMR signals at δ 0.87 and 0.81 ppm indicates that 45 mg of this material is **37** (45% yield) and 218 mg is recovered butyl toluenesulfonate. In addition, 55 mg of 36 was recovered. Data for **37**: TLC, T-3, R_f 0.7; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (t, 3H, J = 7.4 Hz, $-CH_3$), 1.09 (s, 3H, H-18), 2.74−2.93 (m, 2H, H-6), 3.16−3.21 (m, 1H, OCH$_2$), 3.57−3.63 (m, 1H, OCH$_2$), 3.87−3.97 (m, 4H, 17-ketal), 4.25 (dd, 1H, J = 5.7, 2.7 Hz, H-11), 5.03 (s, 2H, OBn), 6.69 (d, 1H, J = 2.7 Hz, H-4), 6.76

(dd, 1H, J = 8.6, 2.7 Hz, H-2), 7.06 (d, 1H, J = 8.6 Hz, H-1), 7.31−7.45 (m, 5H, Ar−H).

3-Benzyloxy-11β-butoxyestra-1,3,5(10)-trien-17-one (38). A solution of impure **37** from the previous step, 200 mg (1.05 mmol) of *p*TsOH in acetone (15 mL), and 20 drops of H_2O was stirred and heated at 70 °C for 1 h. The reaction mixture was allowed to cool to room temperature, poured into saturated $NaHCO_3$ (200 mL), and extracted with CH_2Cl_2 (2×, 150 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving 217 mg of crude **38** which was used without purification in the following step.

3-Benzyloxy-11β-butoxyestra-1,3,5(10)-trien-17β-ol (39). A solution of the crude **38** from the previous step in anhydrous THF (5 mL) was stirred at −78 °C as 104 mg (2.73 mmol) of $LiAlH_4$ was added. The reaction mixture was stirred at −78 °C for 1 h, quenched with EtOAc (5 mL), poured into saturated aqueous sodium−potassium tartrate (50 mL), and extracted with CH_2Cl_2 (3×, 70 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 21 cm column of silica gel using 2:1 hexanes/EtOAc as eluent gave 31 mg (26%, 3 steps) of **39**. Data for **39**: TLC, T-4, R_f 0.13; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (t, 3H, J = 7.9 Hz, $-CH_3$), 0.99 (s, 3H, H-18), 3.13−3.23 (m, 1H, OCH_2), 3.58−3.64 (m, 1H, OCH_2), 3.70 (t, 1H, J = 7.9 Hz, H-17α), 4.22 (dd, 1H, J = 5.5, 2.6 Hz, H-11), 5.04 (s, 2H, OBn), 6.69 (d, 1H, J = 3.0 Hz, H-4), 6.77 (dd, 1H, J = 8.7, 3.0 Hz, H-2), 7.06 (d, 1H, J = 8.7 Hz, H-1), 7.31−7.49 (m, 5H, Ar−H).

11β-Butoxyestra-1,3,5(10)-triene-3,17β-diol (40, E11-0,4$_{ether}$). A suspension of 31 mg (0.071 mmol) of **39** and 5 mg of 5% Pd−C in EtOH (5 mL) was stirred at room temperature under an atmosphere of H_2 for 24 h. The reaction mixture was filtered through a 1 in. pad of Celite and washed through with EtOAc (50 mL). The filtrate was concentrated in vacuo and purified by flash chromatography on a 2 × 21 cm column of silica gel using 2:1 hexanes/EtOAc as eluent, giving 23 mg of **40**. Further purification by HPLC using system H-6 gave 11 mg (45%) of **40** as a white solid. Data for **40**: TLC, T-3, R_f 0.27; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.82 (t, 3H, J = 7.4 Hz, $-CH_3$), 0.99 (s, 3H, H-18), 2.71−2.91 (m, 2H, H-6), 3.17−3.23 (m, 1H, OCH_2), 3.58−3.64 (m, 1H, OCH_2), 3.70 (t, 1H, J = 8.1 Hz, H-17α), 4.20 (dd, 1H, J = 5.6, 2.7 Hz, H-11), 6.54 (d, 1H, J = 2.6 Hz, H-4), 6.62 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 7.02 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{32}O_3Na$ (M + Na^+) *m/e* 367.2249, found *m/e* 367.2241; HPLC system H-7, t_R = 25 min, and system H-16, t_R = 11 min, >99% pure.

3,17β-Dibenzyloxy-11β-methoxymethylestra-1,3,5(10)-triene (42). A suspension of 150 mg (52 mg, 1.31 mmol) of a 35% dispersion of KH in mineral oil was washed with hexanes (2 mL) and suspended in 1 mL of anhydrous toluene. To this was added a solution of 100 mg (0.21 mmol) of **41**[2] in 1 mL of toluene and the reaction was stirred at room temperature for 30 min. To this was added 80 μL (1.25 mmol) of CH_3I, and stirring was continued for 24 h. Another 200 μL (3.2 mmol) of CH_3I was added, and the reaction was stirred for another 24 h, poured into saturated aqueous NH_4Cl (20 mL), and extracted with CH_2Cl_2 (3×, 30 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 8:1 hexanes/EtOAc as eluent gave 26 mg (25%) of **42**. TLC, T-6, R_f 0.59.

11β-Methoxymethylestra-1,3,5(10)-triene-3,17β-diol (43, E11-1,1$_{ether}$). Compound **43** was prepared from **42** (26 mg, 0.052 mmol) in 5:1 EtOH/EtOAc as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1:1 hexanes/EtOAc gave 13.3 mg of **43**. Further purification by HPLC using system H-5 gave 10.5 mg (63%) of **43** as a white solid. Data for **43**: TLC, T-3, R_f 0.13; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 2.42 (dd, 1H, J = 13.2, 1.9 Hz, H-12β), 2.57−2.60 (m, 1H, H-9), 2.67−2.86 (m, 3H, H-6, 11), 3.22−3.32 (m, 2H, OCH_2), 3.24 (s, 3H, OCH_3), 3.74 (dd, 1H, J = 8.8, 7.6 Hz, H-17α), 6.54 (d, 1H, J = 2.6 Hz, H-4), 6.65 (dd, 1H, J = 8.8, 2.6 Hz, H-2), 7.16 (d, 1H, J = 8.8 Hz, H-1); HRMS (ES$^+$) calcd for $C_{20}H_{32}O_3Na$ (M + Na^+) *m/e* 339.1936, found *m/e* 339.1934; HPLC system H-5, t_R = 12 min, and system H-17, t_R = 7.9 min, >99% pure.

3,17β-Dibenzyloxy-11β-ethoxymethylestra-1,3,5(10)-triene (44). Compound **44** was prepared from **41**[2] (85 mg, 0.18 mmol) and EtI (50 μL, 0.6 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 17:1 hexanes/EtOAc as eluent gave 30 mg (33%) of **44** and 22 mg of recovered **41**. Data for **44**: TLC, T-3, R_f 0.84; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 3H, H-18), 1.19 (t, 3H, J = 6.9 Hz, $-CH_3$), 2.58−2.64 (m, 2H, H-9, 12β), 2.69−2.88 (m, 3H, H-6, 11), 3.30−3.42 (m, 4H, OCH_2), 3.51 (t, 1H, J = 7.9 Hz, H-17α), 4.60 & 4.65 (AB quartet, 2H, J_{AB} = 12.5 Hz, OBn), 5.05 (s, 2H, OBn), 6.70 (d, 1H, J = 2.6 Hz, H-4), 6.81 (dd, 1H, J = 8.7, 2.6 Hz, H-2), 7.23 (d, 1H, J = 2.6 Hz, H-1), 7.28−7.47 (m, 10H, Ar−H).

11β-Ethoxymethylestra-1,3,5(10)-triene-3,17β-diol (45, E11-1,2$_{ether}$). Compound **45** was prepared from **44** (15 mg, 0.029 mmol) as described for **6**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1.5:1 EtOAc/hexanes as eluent gave 7 mg of **45**. Further purification by HPLC using system H-1 gave 5.3 mg (55%) of **45** as a white solid. Data for **45**: TLC, T-1, R_f 0.51; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.16 (t, 3H, J = 7.0 Hz, $-CH_3$), 2.46 (dd, 1H, J = 13.2, 1.5 Hz, H-12β), 2.58 (dd, 1H, J = 10.6, 4.9 Hz, H-9), 2.68−2.86 (m, 3H, H-6, 11), 3.25−3.40 (m, 4H, OCH_2), 3.74 (dd, 1H, J = 9.0, 7.4 Hz, H-17α), 6.54 (d, 1H, J = 2.8 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 2.8 Hz, H-2), 7.17 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{21}H_{30}O_3Na$ (M + Na^+) *m/e* 353.2093, found *m/e* 353.2097; HPLC system H-20, t_R = 8.5 min, and system H-12, t_R = 18.3 min, >99% pure.

3,17β-Dibenzyloxy-11β-propoxymethylestra-1,3,5(10)-triene (46). Compound **46** was prepared from **41**[2] (42 mg, 0.087 mmol) and 1-iodopropane (50 μL, 0.52 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc as eluent gave 17 mg (37%) of **46**. Data for **46**: TLC, T-2, R_f 0.69; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, 3H, $-CH_3$), 1.00 (s, 2H, H-18), 2.57−2.65 (m, 2H, H-9, 12β), 2.69−2.87 (m, 3H, H-6,11), 3.19−3.38 (m, 4H, OCH_2), 3.51 (t, 1H, J = 8.1 Hz, H-17α), 4.57 & 4.64 (AB quartet, 2H, J_{AB} = 12.0 Hz, OBn), 5.03 (s, 2H, OBn), 6.69 (d, 1H, J = 2.7 Hz, H-4), 6.79 (dd, 1H, J = 8.7, 2.7 Hz, H-2), 7.22 (d, 1H, J = 8.7 Hz, H-1), 7.29−7.46 (m, 10H, Ar−H).

11β-Propoxymethylestra-1,3,5(10)-triene-3,17β-diol (47, E11-1,3$_{ether}$). Compound **47** was prepared from **46** (15 mg, 0.29 mmol) as described for **6**. Purification by HPLC using system H-1 gave 7 mg (71%) of **47** as a white solid. Data for **47**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (t, 3H, J = 7.4 Hz, $-CH_3$), 0.92 (s, 3H, H-18), 2.58−2.87 (m, 4H, H-6, 9, 11), 3.19−3.37 (m, 4H, OCH_2), 3.71−3.77 (m, 1H, H-17α), 6.55 (d, 1H, J = 2.6 Hz, H-4), 6.65 (dd, 1H, J = 8.3, 2.6 Hz, H-2), 7.19 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES$^+$) calcd for $C_{21}H_{32}O_3Na$ (M + Na^+) *m/e* 367.2249, found *m/e* 367.2241; HPLC system H-20, t_R = 13.1 min, and system H-18, t_R = 6.8 min, >99% pure.

3,17β-Dibenzyloxy-11β-butoxymethylestra-1,3,5(10)-triene (48). Compound **48** was prepared from **41**[2] (100 mg, 0.21 mmol) and iodobutane (142 μL, 1.25 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 8:1 hexanes/EtOAc as eluent gave 95 mg (85%) of **48**: TLC, T-6, R_f 0.66.

11β-Butoxymethylestra-1,3,5(10)-triene-3,17β-diol (49, E11-1,4$_{ether}$). Compound **49** was prepared from **48** (95 mg, 0.18 mmol) as described for **40**. Purification by flash chromatography on a 2 × 27 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 41 mg of **49**. Further purification by HPLC using system H-1 gave 32 mg (50%) of **49** as a white solid. Data for **49**: TLC, T-3, R_f 0.27; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (t, 3H, J = 7.3 Hz, $-CH_3$), 0.91 (s, 3H, H-18), 2.45 (dd, 1H, J = 13.2, 2.0 Hz, H-12β), 2.58 (dd, 1H, J = 10.5, 4.9 Hz, H-9), 2.68−2.86 (m, 3H, H-6, 11), 3.21−3.35 (m, 4H, OCH_2), 3.73 (dd, 1H, J = 8.9, 7.5 Hz, H-17α), 6.54 (d, 1H, J = 2.5 Hz, H-4), 6.64 (dd, 1H, J = 8.5, 2.5 Hz, H-2), 7.17 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{23}H_{34}O_3Na$ (M + Na^+)

m/e 381.2406, found *m/e* 381.2410; HPLC system H-1, t_R = 15 min, and system H-12, t_R = 12.4 min, >99% pure.

11β-(*tert*-Butyldimethylsiloxyethoxy)methyl-3,17β-dibenzyloxyestra-1,3,5(10)-triene (50). Compound **50** was prepared from **41**[2] (57 mg, 0.12 mmol) and (2-bromoethoxy)-*tert*-butyldimethylsilane (500 μL, 2.4 mmol) at 60 °C as described for **42**, except 374 mg (1.42 mmol) of 18-crown-6 was added along with the KH. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc as eluent gave 48 mg (63%) of **50**: TLC, T-2, R_f 0.4.

2-((3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)-methoxy)ethanol (51). A solution of 48 mg (0.074 mmol) of **50** was stirred at room temperature in a 1 M solution of $^nBu_4NF$ in THF (1 mL) for 30 min. The reaction mixture was poured into H_2O (50 mL) and extracted with EtOAc (3×, 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 2:1 hexanes/EtOAc as eluent gave 22 mg (55%) of **51**. Data for **51**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 2.52 (d, 1H, J = 13.0 Hz, H-12β), 2.60 (dd, 1H, J = 10.8, 4.3 Hz, H-9), 2.70−2.86 (m, 3H, H-6, 11), 3.38−3.42 (m, 4H, OCH_2), 3.50 (t, 1H, J = 8.0 Hz, H-17α), 3.65−3.68 (m, 2H, OCH_2), 4.59 (s, 2H, OBn), 5.03 (s, 2H, OBn), 6.70 (d, 1H, J = 2.3 Hz, H-4), 6.80 (dd, 1H, J = 8.6, 2.3 Hz, H-2), 7.22 (d, 1H, J = 8.6 Hz, H-1), 7.29−7.45 (m, 10H, Ar−H).

3,17β-Dibenzyloxy-11β-(methanesulfonyloxyethoxymethyl)estra-1,3,5(10)-triene (52). A solution of **22** mg (0.041 mmol) of **51** and 32 μL (0.41 mmol) of MsCl in pyridine (2 mL) was allowed to stand at 4 °C overnight. The reaction mixture was poured into H_2O (30 mL) and extracted with CH_2Cl_2 (3×, 30 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 2:1 hexanes/EtOAc gave 24 mg (96%) of **52**. Data for **52**: TLC, T-3, R_f 0.40; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.99 (s, 3H, H-18), 2.55 (dd, 1H, J = 13.1, 1.6 Hz, H-12β), 2.60 (dd, 1H, J = 11.0, 4.8 Hz, H-9), 2.71−2.85 (m, 3H, H-6, 11), 2.95 (s, 3H, $-CH_3$), 3.36−3.45 (m, 2H, OCH_2), 3.51 (t, 1H, J = 7.9 Hz, H-17α), 3.55−3.58 (m, 2H, OCH_2), 4.30−4.33 (m, 2H, OCH_2), 4.57 (s, 2H, OBn), 5.04 (s, 2H, OBn), 6.70 (d, 1H, J = 2.7 Hz, H-4), 6.80 (dd, 1H, J = 8.7, 2.7 Hz, H-2), 7.19 (d, 1H, J = 8.7 Hz, H-1), 7.29−7.45 (m, 10H, Ar−H).

3,17β-Dibenzyloxy-11β-(2-fluoroethoxymethyl)estra-1,3,5(10)-triene (53). A solution of 24 mg (0.039 mmol) of **52** was stirred and heated at 70 °C in a 1 M solution of $^nBu_4NF$ in THF (1 mL) for 30 min. The reaction mixture was cooled to room temperature, poured into H_2O (30 mL), and extracted with CH_2Cl_2 (3×, 30 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 4:1 hexanes/EtOAc as eluent gave 15 mg of **53**. Data for **53**: TLC, T-3, R_f 0.69; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.00 (s, 3H, H-18), 2.57−2.86 (m, 5H, H-6, 9, 11, 12β), 3.40−3.43 (m, 2H, OCH_2), 3.48−3.53 (m, 1H, H-17α, OCH_2), 3.57−3.61 (m, 1H, OCH_2), 4.45−4.65 (m, 2H, FCH_2), 4.58 & 4.63 (AB quartet, 2H, J_{AB} = 12.2 Hz, OBn), 5.03 (s, 2H, OBn), 6.69 (d, 1H, J = 2.9 Hz, H-4), 6.80 (dd, 1H, J = 8.6, 2.9 Hz, H-2), 7.22 (d, 1H, J = 2.9 Hz, H-1), 7.29−7.45 (m, 10H, Ar−H).

11β-(2-Fluoroethoxymethyl)estra-1,3,5(10)-triene-3,17β-diol (54, E11-1,2F$_{ether}$). Compound **54** was prepared from **53** (15 mg, 0.028 mmol) in EtOH (5 mL) and EtOAc (1 mL) as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1:1 hexanes/EtOAc gave 7.5 mg of **54**. Further purification by HPLC using system H-5 gave 5.4 mg (54%) of **54** as a white solid. Data for **54**: TLC, T-3, R_f 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (s, 3H, H-18), 2.46 (dd, 1H, J = 13.5, 1.7 Hz, H-12β), 2.59 (dd, 1H, J = 10.7, 4.7 Hz, H-9), 2.65−2.82 (m, 2H, H-6), 2.84−2.91 (m, 1H, H-11), 3.35−3.44 (m, 2H, OCH_2), 3.74 (dd, 1H, J = 8.8, 7.6 Hz, H-17α), 4.50 (dt, 2H, J = 47.5, 4.2, 4.2 Hz, $-CH_2F$), 6.54 (d, 1H, J = 2.7 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 2.7 Hz, H-2), 7.17 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{21}H_{29}FO_3Na$ (M + Na$^+$) *m/e* 371.1998, found *m/e* 371.1988; HPLC system H-5, t_R = 14.5 min, and system H-12, t_R = 14.5 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-methoxyethyl)estra-1,3,5(10)-triene (55). Compound **55** was prepared from **24** (50 mg, 0.10 mmol) and CH_3I (50 μL, 0.80 mmol) as described for **42**. Purification by flash chromatography using 8:1 hexanes/EtOAc as eluent gave 51 mg (100%) of **55**: TLC, T-6, R_f 0.73.

11β-(2-Methoxyethyl)estra-1,3,5(10)-triene-3,17β-diol (56, E11-2,1$_{ether}$). Compound **56** was prepared from **55** (20 mg, 0.039 mmol) as described for **6**. Purification by HPLC using system H-5 gave 7.2 mg (56%) of **56** as a white solid. Data for **56**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3H, H-18), 2.55−2.85 (m, 4H, H-6, 9, 11), 3.32 (s, 3H, OCH_3), 3.45−3.46 (m, 2H, OCH_2), 3.74 (t, 1H, J = 8.8, 7.5 Hz, H-17α), 6.55 (d, 1H, J = 2.7 Hz, H-4), 6.65 (d, 1H, J = 8.4, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{21}H_{30}O_3Na$ (M + Na$^+$) *m/e* 353.2093, found *m/e* 353.2094; HPLC system H-21, t_R = 12.9 min, and system H-12, t_R = 22.5 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-ethoxyethyl)estra-1,3,5(10)-triene (57). Compound **57** was prepared from **24** (32 mg, 0.064 mmol) and EtI (50 μL, 0.62 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 20:1 hexanes/EtOAc gave 10 mg (29%) of **57**. Data for **57**: TLC, T-6, R_f 0.79; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.05 (s, 3H, H-18), 1.20 (t, 3H, J = 7.0 Hz, $-CH_3$), 2.27 (d, 1H, J = 13.2 Hz, H-12β), 2.54−2.62 (m, 2H, H-9, 11), 2.69−2.87 (m, 2H, H-6), 3.39−3.55 (m, 5H, H-17α, OCH_2), 4.60 (s, 2H, OBn), 5.04 (s, 2H, OBn), 6.69 (d, 1H, J = 2.7 Hz, H-4), 6.80 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.12 (d, 1H, J = 8.5 Hz, H-1), 7.28−7.46 (m, 10H, Ar−H).

11β-(2-Ethoxyethyl)estra-1,3,5(10)-triene-3,17β-diol (58, E11-2,2$_{ether}$). Compound **58** was prepared from **57** (10 mg, 0.019 mmol) as described for **6**. Purification by HPLC using system H-1 gave 4.6 mg (70%) of **58**. Data for **58**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (s, 3H, H-18), 1.20 (t, 3H, J = 6.9 Hz, $-CH_3$), 2.52−2.62 (m, 2H, H-9, 11), 2.66−2.84 (m, 2H, H-6), 3.39−3.55 (m, 4H, OCH_2), 3.73 (dd, 1H, J = 8.9, 7.2 Hz, H-17α), 6.54 (d, 1H, J = 2.7 Hz, H-4), 6.63 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{32}O_3Na$ (M + Na$^+$) *m/e* 367.2249, found *m/e* 367.2242; HPLC system H-20, t_R = 9.2 min, and system H-12, t_R = 16.6 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-propoxyethyl)estra-1,3,5(10)-triene (59). Compound **59** was prepared from **24** (60 mg, 0.12 mmol) and 1-iodopropane (50 μL, 0.51 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc as eluent gave 29 mg (44%) of **59**: TLC, T-2, R_f 0.71.

11β-(2-Propoxyethyl)estra-1,3,5(10)-triene-3,17β-diol (60, E11-2,3$_{ether}$). Compound **60** was prepared from **59** (15 mg, 0.028 mmol) as described for **6**. Purification by HPLC using system H-1 gave 5 mg (50%) of **60** as a white solid. Data for **60**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (t, 3H, J = 7.5 Hz, $-CH_3$), 0.96 (s, 3H, H-18), 2.52−2.83 (m, 4H, H-6, 9, 11), 3.30−3.59 (m, 4H, OCH_2), 3.73 (dd, 1H, J = 9.0, 7.1 Hz, H-17α), 6.53 (d, 1H, J = 2.8 Hz, H-4), 6.62 (dd, 1H, J = 8.4, 2.8 Hz, H-2), 7.08 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{23}H_{34}O_3Na$ (M + Na$^+$) *m/e* 381.2406, found *m/e* 381.2408; HPLC system H-20, t_R = 11.2 min, and system H-12, t_R = 12.2 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-isopropoxyethyl)estra-1,3,5-(10)-triene (61). A suspension of 570 mg (200 mg, 5.0 mmol) of a 35% dispersion of KH in mineral oil was washed in a 25 mL pear-shaped flask with hexanes (2 mL). To this was added a solution of 1.39 g (5.25 mmol) of 18-crown-6 and 380 μL (4.96 mmol) of anhydrous iPrOH in anhydrous toluene (4 mL). The reaction was stirred at room temperature for 10 min and then a solution of **162** mg (0.250 mmol) of **25** in toluene (4 mL) was added. The reaction was stirred and heated at 80 °C for 2 h, allowed to cool to room temperature, poured into H_2O (100 mL), and extracted with EtOAc (3×, 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 10:1 hexanes/EtOAc as eluent gave

130 mg (96%) of **61**. Data for **61**: TLC, T-6, R_f 0.66; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.03 (s, 3H, H-18), 1.13 (d, 3H, J = 5.7 Hz, $-CH_3$), 1.14 (d, 3H, J = 5.7 Hz, $-CH_3$), 2.25 (dd, 1H, J = 13.5, 1.1 Hz, H-12β), 2.52–2.58 (m, 2H,H-9, 11), 2.68–2.84 (m, 2H, H-6), 3.39–3.57 (m, 4H, H-11, 17α, OCH_2), 4.58 (s, 2H, OBn), 5.03 (s, 2H, OBn), 6.67 (d, 1H, J = 2.5 Hz, H-4), 6.78 (dd, 1H, J = 8.6, 2.5 Hz, H-2), 7.10 (d, 1H, J = 8.6 Hz, H-1), 7.28–7.45 (m, 10H, Ar–H).

11β-(2-Isopropoxyethyl)estra-1,3,5(10)-triene-3,17β-diol (62, E11-2,iPr$_{ether}$). Compound **62** was prepared from **61** (17 mg, 0.032 mmol) in EtOH (4 mL) as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 5.8 mg of **62**. Further purification by HPLC using system H-4 gave 4.4 mg (38%) of **62** as a white solid. Data for **62**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.95 (s, 3H, H-18), 1.12 (d, 3H, J = 6.1 Hz, $-CH_3$), 1.14 (d, 3H, J = 6.1 Hz, $-CH_3$), 2.13 (dd, 1H, J = 13.5, 1.8 Hz, H-12β), 2.52–2.61 (m, 2H, H-9, 11), 2.66–2.80 (m, 2H, H-6), 3.37–3.42 (m, 1H, OCH_2), 3.47–3.56 (m, 3H, OCH_2, $-CH(CH_3)_2$), 3.72 (t, 1H, J = 8.2 Hz, H-17α), 6.52 (d, 1H, J = 2.8 Hz, H-4), 6.63 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.07 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{23}H_{34}O_3Na$ (M + Na$^+$) m/e 381.2406, found m/e 381.2413; HPLC system H-4, t_R = 15.5 min, and system H-12, t_R = 12.3 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-*tert*-butoxyethyl)estra-1,3,5-(10)-triene (63). A mixture of 11 mg (0.017 mmol) of **25**, 44 mg (0.16 mmol) of 18-crown-6, and 18 mg (0.16 mmol) of KOtBu in anhydrous toluene (1 mL) was stirred and heated at 80 °C for 4 h. The reaction mixture was cooled to room temperature, poured into H_2O (30 mL), and extracted with CH_2Cl_2 (3×, 30 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography on a 2 × 21 cm column of silica gel using 8:1 hexanes/EtOAc as eluent gave 3.2 mg (35%) of **63**. Data for **63**: TLC, T-4, R_f 0.62; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.04 (s, 3H, H-18), 1.17 (s, 9H, tBu), 2.55 (dd, 1H, J = 13.5, 1.2 Hz, H-12β), 2.51–2.58 (m, 2H, H-9, 11), 2.68–2.82 (m, 2H, H-6), 3.30–3.48 (m, 2H, OCH_2), 3.48 (t, 1H, J = 7.9 Hz, H-17α), 4.59 (s, 2H, OBn), 5.03 (s, 2H, OBn), 6.66 (d, 1H, J = 2.6 Hz, H-4), 6.78 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 7.11 (d, 1H, J = 8.4 Hz, H-1), 7.27–7.44 (m, 10H, Ar–H).

11β-(2-t-Butoxyethyl)estra-1,3,5(10)-triene-3,17β-diol (64, E11-2,tBu$_{ether}$). Compound **64** was prepared from **63** (3.2 mg, 0.0058 mmol) as described for **40**. Purification by HPLC using system H-1 gave 1 mg (40%) of **64** after crystallization from acetone–petroleum ether. Data for **64**: TLC, T-5, R_f 0.7; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3H, H-18), 1.16 (s, 9H, tBu), 2.13 (dd, 1H, J = 13.4, 1.7 Hz, H-12β), 2.51–2.59 (m, 2H, H-9, 11), 2.65–2.80 (m, 2H, H-6), 3.38 (m, 2H, OCH_2), 3.71 (dd, 1H, J = 9.0, 7.1 Hz, H-17α), 6.51 (d, 1H, J = 2.7 Hz, H-4), 6.63 (dd, 1H, J = 8.4, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{24}H_{36}O_3Na$ (M + Na$^+$) m/e 395.2562, found m/e 395.2563; HPLC system H-1, t_R = 15 min, and system H-12, t_R = 11.8 min, >99% pure.

3,17β-Dibenzyloxy-11β-(2-(2-fluoroethoxy)ethyl)estra-1,3,5(10)-triene (65). Compound **65** was prepared from **25** (38 mg, 0.59 mmol) and 2-fluoroethanol (69 μL, 2.3 mmol) as described for **61**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 6:1 hexanes/EtOAc gave 30 mg (94%) of **65**: TLC, T-4, R_f 0.5.

11β-(2-(2-Fluoroethoxy)ethyl)estra-1,3,5(10)-triene-3,17β-diol (66, E11-2,2F$_{ether}$). Compound **66** was prepared from **65** (30 mg, 0.056 mmol) in EtOH (4 mL) and EtOAc (4 mL) as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 11 mg of **66**. Further purification by HPLC using system H-5 gave 8.2 mg (41%) of **66** as a white solid. Data for **66**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3H, H-18), 2.14 (dd, 1H, J = 13.4, 1.7 Hz, H-12β), 2.55 (dd, 1H, J = 11.1, 4.6 Hz, H-9), 2.59–2.64 (m, 1H, H-11), 2.66–2.84 (m, 2H, H-6), 3.53–3.57 (m, 2H, OCH_2), 3.58–3.62 & 3.65–3.69 (m, 2H, J_{HF} = 29.9 Hz, CH_2F), 3.72 (dd, 1H, J = 8.9, 7.5 Hz, H-17α), 4.54 (dt, 2H, J = 4.1, 4.1 Hz, J_{HF} = 47.8 Hz, CH_2F), 6.54 (d, 1H, J = 2.7 Hz, H-4), 6.64 (dd, 1H, J = 8.3, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES$^+$) calcd for $C_{20}H_{31}FO_3Na$ (M + Na$^+$) m/e 385.2155, found m/e 385.2152; HPLC system H-5, t_R = 16.5 min, and system H-12, t_R = 13.1 min, >99% pure.

3,17β-Dibenzyloxy-11β-(3-methoxypropyl)estra-1,3,5-(10)-triene (68). Compound **68** was prepared from **67**[2] (42 mg, 0.082 mmol) and CH_3I (50 μL, 0.8 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc as eluent gave 17 mg (39%) of **68**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.03 (s, 3H, H-18), 2.60–2.87 (m, 2H, H-6), 3.29–3.33 (m, 2H, OCH_2) 3.31 (s, 3H, OCH_3), 3.49 (t, 1H, J = 7.9 Hz, H-17α), 4.59 (s, 2H, OBn), 5.04 (s, 2H, OBn), 6.70 (d, 1H, J = 2.8 Hz, H-4), 6.80 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.07 (d, 1H, J = 8.5 Hz, H-1), 7.29–7.47 (m, 10H, Ar–H).

11β-(3-Methoxypropyl)estra-1,3,5(10)-triene-3,17β-diol (69, E11-3,1$_{ether}$). Compound **69** was prepared from **68** (15 mg, 0.028 mmol) as described for **6**. Purification by HPLC using system H-1 gave 6 mg (61%) of **69** as a white solid. Data for **69**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 3H, H-18), 2.67–2.83 (m, 2H, H-6), 3.28–3.33 (m, 2H, OCH_2), 3.30 (s, 3H, OCH_3), 3.72 (t, 1H, J = 7.7 Hz, H-17α), 6.55 (d, 1H, J = 2.7 Hz, H-4), 6.65 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.04 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{32}O_3Na$ (M + Na$^+$) m/e 367.2249, found m/e 367.2254; HPLC system H-20, t_R = 9 min, and system H-15, t_R = 10 min, >99% pure.

3,17β-Dibenzyloxy-11β-(3-ethoxypropyl)estra-1,3,5(10)-triene (70). Compound **70** was prepared from **67** (50 mg, 0.098 mmol) and EtI (100 μL, 1.2 mmol) as described for **42**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc as eluent gave 28 mg (53%) of **70**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.03 (s, 3H, H-18), 1.19 (t, 3H, J = 6.9 Hz, $-CH_3$), 2.65–2.84 (m, 2H, H-6), 3.33–3.37 (m, 2H, OCH_2), 3.42–3.51 (m, 3H, H-17α, OCH_2), 4.60 (s, 2H, OBn), 5.04 (s, 2H, OBn), 6.71 (d, 1H, J = 2.5 Hz, H-4), 6.80 (dd, 1H, J = 8.6, 2.5 Hz, H-2), 7.08 (d, 1H, J = 8.6 Hz, H-1), 7.28–7.47 (m, 10H, Ar–H).

11β-(3-Ethoxypropyl)estra-1,3,5(10)-triene-3,17β-diol (71, E11-3,2$_{ether}$). Compound **71** was prepared from **70** (15 mg, 0.28 mmol) as described for **6**. Purification by HPLC using system H-1 gave 5 mg (50%) of **71** as a white solid. Data for **71**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 3H, H-18), 1.19 (t, 3H, J = 6.9 Hz, $-CH_3$), 2.67–2.85 (m, 2H, H-6), 3.34–3.37 (m, 2H, OCH_2), 3.42–3.47 (m, 2H, OCH_2), 3.72 (dd, 1H, J = 8.9, 7.0 Hz, H-17α), 6.55 (d, 1H, J = 2.8 Hz, H-4), 6.65 (dd, 1H, J = 8.4, 2.8 Hz, H-2), 7.04 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES$^+$) calcd for $C_{22}H_{34}O_3Na$ (M + Na$^+$) m/e 381.2406, found m/e 381.2403; HPLC system H-20, t_R = 10.2 min, and system H-15, t_R = 8.8 min, >99% pure.

3-(3,17β-Dibenzyloxyestra-1,3,5(10)-trien-11β-yl)propyl Toluenesulfone (72). Compound **72** was prepared from **67** (242 mg, 0.473 mmol) as described for **25**. Purification by flash chromatography on a 3 × 17 cm column of silica gel using 4:1 hexanes/EtOAc as eluent gave 132 mg (42%) of **72**: TLC, T-6, R_f 0.4; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 2.20 (d, 1H, J = 13.9 Hz, H-12β), 2.36–2.41 (m, 1H, H-11), 2.41 (s, 3H, $-CH_3$), 2.52 (dd, 1H, J = 10.7, 4.3 Hz, H-9), 2.69–2.82 (m, 2H, H-6), 3.45 (t, 1H, J = 7.9 Hz, H-17α), 3.95–3.97 (m, 2H, $-OCH_2$), 4.57 & 4.54 (AB quartet, 2H, J_{AB} = 12.2 Hz, OBn), 5.04 (s, 2H, OBn), 6.69 (d, 1H, J = 2.5 Hz, H-4), 6.77 (dd, 1H, J = 8.4, 2.5 Hz, H-2), 6.99 (d, 1H, J = 8.4 Hz, H-1), 7.29–7.45 (m, 12H, Ar–H), 7.72 (d, 2H, J = 8.3 Hz, Ar–H)

3,17β-Dibenzyloxy-11β-(3-isopropoxypropyl)estra-1,3,5-(10)-triene (73). Compound **73** was prepared from **72** (30 mg, 0.045 mmol) and iPrOH (69 μL, 0.90 mmol) as described for **61**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 10:1 hexanes/EtOAc as eluent gave 12 mg (50%) of **73**: TLC, T-6, R_f 0.71;

11β-(3-Isopropoxypropyl)estra-1,3,5(10)-triene-3,17β-diol (74, E11-3,iPr$_{ether}$). Compound **74** was prepared from **73** (12 mg, 0.023 mmol) in EtOH (4 mL) and EtOAc (2 mL) as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 6.8 mg of **74**. Further purification by HPLC using system H-1 gave 6.1 mg (73%) of **74** as a white solid. Data for **74**:

TLC, T-3, R_f 0.23; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 1.11 (d, 3H, J = 6.1 Hz, $-CH_3$), 1.12 (d, 3H, J = 6.1 Hz, $-CH_3$), 2.23 (dd, 1H, J = 13.5, 1.7 Hz, H-12β), 2.42−2.48 (m, 1H, H-11), 2.54 (dd, 1H, J = 10.4, 4.2 Hz, H-9), 2.67−2.83 (m, 2H, H-6), 3.27−3.36 (m, 2H, OCH_2), 3.51 (septet, 1H, J = 6.1 Hz, $CH(CH_3)_2$), 3.71 (dd, 1H, J = 9.0, 7.5 Hz, H-17α), 6.54 (d, 1H, J = 2.5 Hz, H-4), 6.63 (dd, 1H, J = 8.5, 2.5 Hz, H-2), 7.04 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{24}H_{36}O_3$-Na (M + Na$^+$) *m/e* 395.2562, found *m/e* 395.2554; HPLC system H-1, t_R = 13.2 min, and system H-12, t_R = 12.7 min, >99% pure.

3,17β-Dibenzyloxy-11β-(3-*tert*-butoxypropyl)estra-1,3,5-(10)-triene (75). Compound **75** was prepared from **72** (77 mg, 0.116 mmol) and tBuOH (218 μL, 2.31 mmol) as described for **61**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 12:1 hexanes/EtOAc gave 35 mg (53%) of **75**. Data for **75**: TLC, T-4, R_f 0.62; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 3H, H-18), 1.17 (s, 9H, tBu), 2.39 (dd, 1H, J = 13.7, 1.3 Hz, H-12β), 2.42−2.48 (m, 1H, H-11), 2.54 (dd, 1H, J = 10.6, 4.1 Hz, H-9), 2.69−2.86 (m, 2H, H-6), 3.20−3.28 (m, 2H, OCH_2), 3.48 (t, 1H, J = 7.9 Hz, H-17α), 4.57 & 4.60 (AB quartet, 2H, J_{AB} = 12.2 Hz, OBn), 5.03 (s, 2H, OBn), 6.70 (d, 1H, J = 2.8 Hz, H-4), 6.80 (dd, 1H, J = 8.6, 2.8 Hz, H-2), 7.09 (d, 1H, J = 8.6 Hz, H-1), 7.28−7.46 (m, 10H, Ar−H).

11β-(3-*tert*-Butoxypropyl)estra-1,3,5(10)-triene-3,17β-diol (76, E11-3,17Bu$_{ether}$). Compound **76** was prepared from **75** (10 mg, 0.019 mmol) as described for **40**. Purification by flash chromatography on a 1 × 10 cm column of silica gel using 1:1 hexanes/EtOAc as eluent gave 7.1 mg of **76**. Further purification by HPLC using system H-1 gave 4.5 mg (61%) of **76** as a white solid. Data for **76**: TLC, T-4, 0.06; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 1.16 (s, 9H, tBu), 2.24 (dd, 1H, J = 13.6, 1.8 Hz, H-12β), 2.42−2.48 (m, 1H, H-11), 2.54 (dd, 1H, J = 10.3, 4.6 Hz, H-9), 2.67−2.82 (m, 2H, H-6), 3.20−3.28 (m, 2H, OCH_2), 3.71 (t, 1H, J = 8.4 Hz, H-17α), 6.54 (d, 1H, J = 2.8 Hz, H-4), 6.64 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.04 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES$^+$) calcd for $C_{28}H_{38}O_3Na$ (M + Na$^+$) *m/e* 409.2719, found *m/e* 409.2719; HPLC system H-1, t_R = 16.2 min, and system H-12, t_R = 12.9 min, >99% pure.

3,17β-Dibenzyloxy-11β-(3-(2-fluoroethoxy)propyl)estra-1,3,5(10)-triene (77). Compound **77** was prepared from **72** (69 mg, 0.10 mmol) and 2-fluoroethanol (61 μL, 1.0 mmol) as described for **61**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 6:1 hexanes/EtOAc as eluent gave 24 mg (42%) of **77**. Data for **77**: TLC, T-2, R_f 0.28; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.01 (s, 3H, H-18), 2.34 (dd, 1H, J = 13.5, 1.4 Hz, H-12β), 2.42−2.47 (m, 1H, H-11), 2.54 (dd, 1H, J = 10.6, 4.0 Hz, H-9), 2.69−2.86 (m, 2H, H-6), 3.40−3.44 (m, 2H, OCH_2), 3.48 (dd, 1H, J = 8.5, 7.3 Hz, H-17α), 3.59−3.61 & 3.66−3.68 (m, 2H, J_{HF} = 29.5, OCH_2), 4.59−4.48 & 4.58−4.60 (m, 2H, J_{HF} = 47.7 Hz, CH_2F), 4.58 (s, 2H, OBn), 5.03 (s, 2H, OBn), 6.69 (d, 1H, J = 2.7 Hz, H-4), 6.79 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.07 (d, 1H, J = 8.5 Hz, H-1), 7.28−7.45 (m, 10H, Ar−H).

11β-(3-(2-Fluoroethoxy)propyl)estra-1,3,5(10)-triene-3,17β-diol (78, E11-3,2F$_{1ether}$). Compound **78** was prepared from **77** (24 mg, 0.044 mmol) in EtOH (2 mL) and EtOAc (2 mL) as described for **40**. Purification by flash chromatography on a 2 × 17 cm column of silica gel using 1.5:1 hexanes/EtOAc as eluent gave 13 mg of **78**. Further purification by HPLC using system H-4 gave 11 mg (66%) of **78** as a white solid. Data for **78**: TLC, T-5, R_f 0.42; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3H, H-18), 2.24 (dd, 1H, J = 13.7, 1.4 Hz, H-12β), 2.44−2.50 (m, 1H, H-11), 2.56 (dd, 1H, J = 11.0, 5.5 Hz, H-9), 2.68−2.85 (m, 2H, H-6), 3.42−3.45 (m, 2H, OCH_2), 3.60−3.62 & 3.66−3.69 (m, 2H, J_{HF} = 29.4 Hz, OCH_2), 3.73 (dd, 1H, J = 9.0, 7.1 Hz, H-17α), 4.47−4.49 & 4.59−4.61 (m, 2H, J_{HF} = 47.7 Hz, CH_2F), 6.56 (d, 1H, J = 2.8 Hz, H-4), 6.65 (dd, 1H, J = 8.6, 2.8 Hz, H-2), 7.04 (d, 1H, J = 8.6 Hz, H-1); HRMS (ES$^+$) calcd for $C_{25}H_{33}FO_3Na$ (M + Na$^+$) *m/e* 399.2311, found *m/e* 399.2322; HPLC system H-4, t_R = 14 min, and system H-12, t_R = 12.1 min, >99% pure.

Competitive Binding to Rat Cytosolic ERα, Human LBD-ERα, and Human LBD-ERα ERβ. Binding affinities relative to E_2 were performed in incubations with the ER (ERa^{24}) in rat uterine cytosol. Female Sprague−Dawley rats were castrated and sacrificed 24 h later. The uterus was removed, homogenized in ice-cold TEGDMo buffer (10 mM Tris, 1.5 mM Na_2EDTA, 10% (v/v) glycerol, 1.0 mM dithiothreitol, 25 mM sodium molybdate, pH 7.4 at 4 °C), and centrifuged at 105 000*g* for 45 min at 4 °C. The supernatant (cytosol) was frozen on dry ice and stored at −80 °C until assay. For assay, the cytosol was defrosted, diluted, and incubated with 1 nM [^{3}H]E_2 in the presence and absence of nonradioactive E_2, estrone, or the E_2-analogues over a range of concentrations from 10^{-12} to 10^{-6} M. Incubations were carried out on ice overnight, and bound radioactivity was separated from free by adsorption with dextran-coated charcoal and quantified by counting.[39] Relative binding affinity (RBA) was determined by analysis of the displacement curves by the curve-fitting program Prism. For comparison, we measured the binding of all E_2-11β-ethers to the LBD of human ERα (M_{295}−V_{595})[23] and human ERβ (M_{244}−Q_{530}).[22] The assay was performed overnight at room temperature in competition with [^{3}H]E_2 in lysates of *Escherichia coli* in which the LBDs are expressed as described.[40] The results, as RBAs compared to E_2, of all receptor studies shown in Table 2, are from at least three separate experiments performed in duplicate. RBAs represent the ratio of the EC_{50} of E_2 to that of the steroid analogue × 100 using the curve-fitting program Prism to determine the EC_{50}.

Estrogenic Potency in Ishikawa Cells. The estrogenic potency of the E_2-analogues was determined in an estrogen bioassay, the induction of AlkP in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates, as we have previously described.[4] The cells are grown in phenol red free medium with estrogen-depleted (charcoal stripped) bovine serum in the presence or absence of varying amounts of the steroids, across a dose range of at least 6 orders of magnitude. After 3 days, the cells are washed, frozen, thawed, and then incubated with 5 mM *p*-nitrophenyl phosphate, a chromogenic substrate for the AlkP enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of *p*-nitrophenol at 405 nm. For antagonists, the effect (K_i) of each compound tested at a range from 10^{-6} to 10^{-12} M was measured for the inhibition of the action of 10^{-9} M E_2 (EC_{50} ~ 0.2 nM). Each compound was analyzed in at least three separate experiments performed in duplicate. The K_i and RSA (RSA = ratio of $1/EC_{50}$ of the steroid analogue to that of E_2 × 100) were determined using the curve-fitting program Prism.

ERα-, ERβ-, and ERE-Transfected JAR Cells. The specificity of the antiestrogenic activity relative to the ER subtypes, ERα and ERβ, was determined in the human choriocarcinoma JAR cell line transfected with plasmids containing a consensus estrogen response element (ERE) fused to a firefly luciferase reporter gene and separately with either the expression vectors for human ERα or human ERβ exactly as we described.[5] After transfection, the medium was changed to phenol red free RPMI containing 10% dextran-coated charcoal-treated calf serum. Twenty-four hours later, E_2 or E11-2,2$_{ether}$ (10^{-12}−10^{-6} M) + E_2 (1 nM) were added and the cells were incubated for 12 h at 37 °C in 5% CO_2. The cells were harvested in 10 mM Tris-HCl/10 mM EDTA/150 mM NaCl and centrifuged for 4 min at 4000 rpm, supernatant was removed, and cell pellets were lysed in lysis buffer 2 (Bio-Orbit, Turku, Finland). Luciferase activity was measured using the GenGlow system (Promega, Madison, WI).

Uterotrophic Stimulation in Immature Rats. All animal procedures were approved by the Yale University Institutional Animal Care and Use Committee. The uterotrophic assay was performed in immature rats as described.[41] Female Sprague−Dawley rats (n = 6/group), 21-days-old, were injected subcutaneously daily for 3 days with (total dose) E_2 (20 ng), E11-2,2$_{ether}$ (100 ng and 1 μg), or a combination of the 2 doses of E11-2,2$_{ether}$ + E_2 in 0.1 mL of sesame oil. Control animals

received the vehicle. On the fourth day, the animals were killed, and the uteri were removed, dissected, blotted, and weighed.

Tissue-Selective Effects in Ovariectomized Rats. Sprague−Dawley rats (~250 g) were ovariectomized and starting that evening were injected daily with either 33 ng/kg of E_2 or E11-2,2_{ether} (36 or 180 ng) or vehicle alone (0.1 mL of sesame oil) subcutaneously for 7 days. The following day the animals were killed by exsanguination while under ether anesthesia. Serum was obtained and total cholesterol concentration was determined by a commercial chromogenic assay (Roche Diagnostics, Indianapolis IN). The uteri were dissected and weighed. In a separate study, the ovariectomized rats were injected with vehicle alone (control), E11-2,2_{ether} (180 ng) or E11-2,2_{ether} (180 ng) + ICI 182,780 (250 μg) as above, and serum cholesterol was determined.

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Estrogen to Antiestrogen with a Single Methylene Group Resulting in an Unusual Steroidal Selective Estrogen Receptor Modulator

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Selective estrogen receptor (ER) modulators (SERMs) are important therapeutic agents for breast cancer prevention and treatment. We have synthesized two analogs, E11–2,1 [methyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate] and E11–2,2 [ethyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate], the methyl and ethyl esters of an estradiol analog, substituted in the B ring at C-11β with a carboxymethyl group. The shorter methyl ester, E11–2,1, has high ER affinity and high estrogenic potency in the Ishikawa estrogen cell bioassay, whereas the longer ethyl ester, E11–2,2, has even higher ER affinity, but little or no estrogenic activity. We found that this minor change of one methylene group transforms a potent estrogenic agonist into an antagonist *in vitro* with either ER α or β. In the rat, E11–2,2 acts as a SERM in the uterus, where it inhibits estradiol-induced proliferation, and as an estrogen agonist in the liver and skeleton, where it decreases plasma cholesterol and increases bone growth. The characteristic feature of antiestrogens, including SERMs, is a long and polar side-chain that prevents agonist-induced conformation of helix 12 of ER. E11–2,2 with its short, nonpolar side-chain, lacks this critical structure, presenting the possibility that it might act through a unique mechanism. (*J Clin Endocrinol Metab* 89: 3527–3535, 2004)

IN ADDITION TO the many estrogen agonists, potent antagonists have been developed for therapeutic intervention, including some that are tissue specific and act as either agonists or antagonists depending upon the site of action (1, 2). They have been named selective estrogen receptor (ER) modulators (SERMs). As shown in Fig. 1, SERMS and pure antiestrogens (either steroidal or nonsteroidal) share the common structural feature of a long-chain extension bearing a polar or charged group close to its terminus. Recently, we synthesized an unusual compound that has the properties of a SERM, but is devoid of their characteristic long-chain and polar substituents. We had been synthesizing novel families of locally active "soft" estrogens, for treatment of vaginal dyspareunia associated with menopause or antiestrogen therapy (3, 4). These compounds are esters of carboxylic acid analogs of estradiol (E_2), and the esters are ER ligands capable of estrogenic stimulation, whereas the parent, charged carboxylates, are not. This feature makes them susceptible to hydrolysis by esterases, ubiquitous enzymes that rapidly convert the estrogen esters into their carboxylates, thereby generating inactive metabolites. Because they are metabolically labile, these compounds are estrogenic only in tissues in which they are placed directly; for example, the vagina. Substituents were placed at positions in E_2 that are known to minimally interfere with binding (7α-, 11β-, 15α-, and 16α-). Strangely, some of the derivatives at C-11β exhibited unusual properties: a dramatic disassociation between ER binding and estrogenic action. The methyl ester, E11–2,1 [methyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate; Fig. 1] bound strongly to the ER (Fig. 2A), and as expected, it was a strong agonist in the Ishikawa cell estrogen bioassay (Fig. 2B). In contrast, the ethyl ester, E11–2,2 [ethyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate; Fig. 1] also bound strongly to the ER (slightly better than E11–2,1), but it was almost devoid of activity in the Ishikawa assay (Fig. 2B). There was some minor stimulatory activity at very high concentrations of E11–2,2, but it did not approach the maximum induced with E_2 or E11–2,1.

The Ishikawa cell estrogen bioassay, which was developed in this laboratory, has been widely used because it closely mirrors the *in vivo* behavior of estrogens (5). In most cases there is parallelism between ER affinity and Ishikawa cell potency. Consequently, disassociation between ER binding and biological stimulation is unusual for estrogen agonists, but it is a fundamental property of estrogen antagonists and indicates that E11–2,2 might be one. If so, this seemingly minor modification of a one-carbon methyl ester (E11–2,1) into a two-carbon ethyl ester (E11–2,2) has transformed a relatively strong agonist into an antagonist. However, E11–2,2 is devoid of the long polar side-chain that is a hallmark of an antiestrogen and would be a highly unusual estrogen antagonist. This led us to undertake the study reported in this paper to investigate the possibility that E11–2,2 is an antiestrogen or a SERM.

Materials and Methods

E11–2,1 and E11–2,2 [systematic names are, respectively, methyl (3, 17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate; ethyl (3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate] were synthesized as we

Abbreviations: AlkP, Alkaline phosphatase; E_1, estrone; E11-2,1, methyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate; E11-2,2, ethyl-(3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl)acetate; E_2, estradiol; ER, estrogen receptor; ERE, ER element; LBD, ligand binding domain; MMA, methymethacrylate; SERM, selective ER modulator; THC, tetrahydrochrysene.

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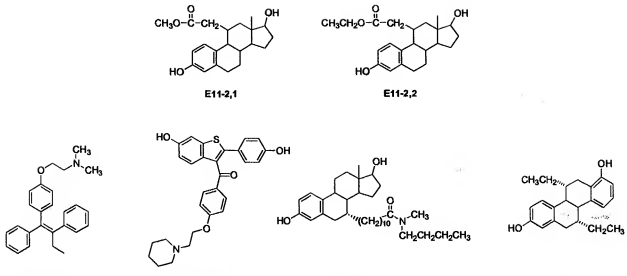

FIG. 1. Structures of E11–2,1 and E11–2,2; the SERMs, tamoxifen and raloxifene; the pure antiestrogen, ICI 164,384; and the ERβ antagonist/ERα agonist, R,R-THC.

previously described (4). Statistical analyses (ANOVA) were performed using PRISM (GraphPad, Inc., San Diego, CA).

Ishikawa cell assay

The Ishikawa cell assay was performed as we previously described (5). In short, the cells were grown in 96-well plates in estrogen-free medium (phenol red free, with charcoal-stripped calf serum) in the presence of (stimulatory assay) test compounds as well as E_2 and estrone (E_1; Steraloids, Inc., Newport, RI) at concentrations that were varied over several log orders. For the antiestrogen assay, a range of concentrations of E11–2,1 or E11–2,2 was added concurrently with 1 nM E_2. The treated cells were grown for 3 d. To determine alkaline phosphatase (AlkP) activity, the cells were frozen, defrosted, and incubated with the chromogenic substrate, *p*-nitrophenylphosphate, at room temperature. The hydrolysis product, *p*-nitrophenol, was measured kinetically at 405 nm. The K_i was determined by comparison with the K_n of E_2 (determined in parallel) using the curve-fitting program PRISM.

ERα, ERβ, and ER element (ERE)-transfected JAR cells

The specificity of the antiestrogenic activity relative to the ER subtypes, ERα and ERβ, was determined in the human choriocarcinoma JAR cell line transfected with plasmids containing a consensus ERE fused to a firefly luciferase reporter gene and separately with the expression vectors for either human ERα or ERβ. JAR cells were routinely cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 0.5% nonessential amino acids (Life Technologies, Inc.), and 1% PEST (100 U penicillin/ml and 100 μg streptomycin/ml). Cells were seeded in six-well plates 24 h before transfection. Transfections using the Mirus Trans IT (Mirus Corp., Madison, WI) reagent were performed as described by the manufacturer in a serum- and antibiotic-free mixture of phenol-red free OptiMEM with 0.75 μg 3×ERE-TATA-Luc reporter (6, 7) and 0.1–0.4 μg pCXN2 human ERα or pCXN2 h-ERβ as indicated (8). The pCXN2 h-ERα (9) and pCXN2 h-ERβ (10) were gifts from Prof. Satoshi Inoue (University of Tokyo, Tokyo, Japan) (11). The ERE-reporting vector was constructed by introducing an *HpaI/BglII* fragment containing 3× ERE-TATA into *SmaI/BglII* of the pGL3-Luc basic vector (Invitrogen, Carlsbad, CA). Medium was changed to a phenol red-free RPMI containing 10% dextran-coated charcoal-treated calf serum, 0.5% nonessential amino acids, and no PEST. After 24 h, E_2 and the indicated concentrations of E11–2,2 or vehicle (0.1% ethanol) were added simultaneously. Cells were incubated for 12 h at 37 C in 5% CO_2. The cells were harvested in 10 mM Tris-HCl/10 mM EDTA/150 mM NaCl and centrifuged for 4 min at 4000 rpm, supernatant was removed, and cell pellets were lysed in Lysis Buffer 2 (Bio-Orbit, Turku, Finland). Luciferase activity was measured using the GenGlow system (Promega, Madison, WI).

In vivo estrogenic/antiestrogenic activity

All animal procedures were approved by the Yale University Institutional animal care and use committee.

Western blot analysis of ERα

ERα was determined by Western blotting, performed essentially as we previously described (7). Ishikawa cells treated separately with vehicle, 10^{-9} or 10^{-8} M E_2, and E11–2,2 were grown for 3 d under conditions described above for the AlkP assay. Afterward the cells were washed three times with PBS and lysed using 1% Nonidet P-40 and 0.1% sodium dodecyl sulfate in the presence of protease inhibitors. Proteins (25 μg/well) were separated by SDS-PAGE on ice using 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were stained with Ponceau Red before the antibody incubation to ensure proper transfer. Immunoblotting was performed after blocking the membranes with 5% powdered milk in water. The blots were incubated first with the ERα monoclonal antibody clone 6F11 (Novocastra, Newcastle, UK) overnight at 4 C. ERα was detected using peroxidase-labeled horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) and Chemiluminescence Reagent Plus (PerkinElmer, Wellesley, MA). The intensity of the signal was analyzed using a digital imaging analysis system (1D Image Analysis software; Scientific Imaging Kodak Co., Rochester, NY). β-Actin was used as an internal control to normalize the amount of protein loaded in the gels.

Uterotrophic stimulation in immature rats

The uterotrophic assay was performed in immature rats as previously described (12). Female Sprague Dawley rats, 22 d old, were injected sc daily for 3 d with E_2 (20 ng, total dose), E11–2,2 (1 and 10 μg), or a mixture of E_2 and E11–2,2. Control animals received vehicle (0.1 ml sesame oil). On the fourth day, animals were killed, and uteri were removed, dissected, blotted, and weighed. Each compound was assayed in two separate experiments, with five or six animals per group in each.

Tissue-selective effects in ovariectomized rats

To determine whether E11–1,2 had tissue-selective effects, ovariectomized, approximately 250-g female Sprague Dawley rats were injected

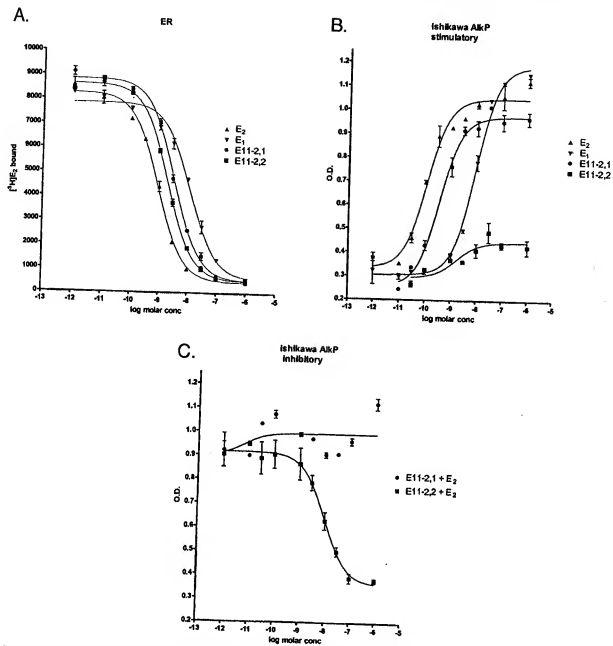

FIG. 2. A, Competition of binding of $[^{125}I]E_2$ to the ER in castrate rat uterine cytosol. B, Estrogenic stimulation of AlkP in Ishikawa cells. C, Inhibition of estrogenic stimulation of AlkP in Ishikawa cells. The cells were stimulated with 10^{-9} M E_2 and the indicated concentration of E11–2,2 or E11–2,1. None of the studies is background subtracted. Note (C) that approximately 10^{-7} M E11–2,2 completely suppresses E_2 stimulation. A and B are drawn from data in representative experiments previously reported (4). *Error bars* show the SEM.

with 400 ng/kg E_2, three different concentrations of E11–2,2 (20, 60, and 600 μg/kg), or vehicle alone (0.1 ml sesame oil) sc for 35 d. The following day the animals were killed by exsanguination while under ether anesthesia.

Cholesterol. Serum was obtained, and the total cholesterol concentration was determined by a commercial chromogenic assay (Roche, Indianapolis IN).

Uteri. The uteri were dissected, weighed, fixed in formalin, and imbedded in paraffin, and 5-μm sections were prepared. Endometrial luminal epithelium and glandular cell height were measured using the Openlab image analysis system (Improvision, Lexington, MA). Cellular height (in micrometers) was based on calibration with an ocular micrometer (×400) on the microscope. Three to six regions were analyzed in each slide.

Bone. The tibia were dissected free of extraneous tissue and then analyzed histomorphometrically as follows. The bones were fixed in 70% ethanol, dehydrated in graded ethanol, and cleared in toluene. The specimens were then infiltrated with increasing concentrations of methylmethacrylate (MMA) and embedded in MMA, as previously described (13). After polymerization, MMA blocks were cut to size, sanded, and polished to the appropriate level. Sections of 4–5 μm were cut, mounted on gelatin-coated slides, and stained with toluidine blue. The bone was analyzed in a blinded manner for standard histomorphometrical measures (14) using the computerized Osteomeasure analysis system (Osteometrics, Atlanta, GA).

The histomorphometric measurements of bone and uterus were performed in tissue sections selected randomly by an independent individual blinded to the treatment groups. The results were compared with control animals receiving sesame oil alone and to the group that was injected with E_2.

In a separate experiment, 250-g ovariectomized rats were injected sc with 400 ng/kg E_2, 600 μg/kg E11–2,2, or vehicle alone (0.1 ml sesame oil) for 8 d. Steroid administration was started immediately after surgery (d 0). For acclimatization, the animals were handled several times a day, every day. On the evening of the seventh day, food was removed; the next morning the animals were restrained, and core body temperature was measured rectally with a rat probe (Physitemp Instruments, Inc., Clifton, NJ). Afterward they were anesthetized with ether, weighed, and killed by exsanguination. Uteri were weighed as described above, dried overnight in an oven at 105 C, and then weighed again to obtain dry weight. Serum cholesterol levels were determined as described above.

Results

Ishikawa cells

The antiestrogenic activity of the two E_2 analogs was determined in the Ishikawa cell bioassay by adding the carboxylic acid ester concurrently with 1 nM E_2. E11–2,1 does not inhibit the estrogenic effect of E_2 on AlkP (Fig. 2C). In contrast, E11–2,2, causes a marked inhibition of estradiol, which at approximately 10^{-7} M reduces estrogenic stimulation to baseline (Fig. 2C). The K_i of E11–2,2 determined in four separate experiments performed in duplicate is 3.9 ± 1.4 nM.

ERα- and ERβ-transfected JAR cells

To determine whether E11–2,2 inhibited the estrogenic action of E_2 in either ER subtype specifically, JAR cells stimulated with 10^{-9} M E_2 and transfected with either ERα or ERβ were concomitantly incubated with varying concentrations of E11–2,2. As shown in Fig. 3, E11–2,2 completely abolished, with approximately the same potency, the estrogenic stimulation of the luciferase reporter in cells containing either ERα or ERβ. Consequently, E11–2,2 acts as an antiestrogen with both ER subtypes.

Effect of E11–2,2 on ERα in Ishikawa cells

Ishikawa cells were grown in the presence of vehicle, 10^{-7} and 10^{-8} M E_2, and 10^{-7} and 10^{-8} M E11–2,2. After 3 d, ERα protein content was determined by Western blotting (Fig. 4); compared with the vehicle control (without added steroid) the levels were: 10^{-8} M E_2, 72%; 10^{-7} M E_2, 71%; 10^{-8} M E11–2,2, 107%; and 10^{-7} M E11–2,2, 104%.

Uterotrophic effect in the immature rat

The estrogenic and antiestrogenic actions of E11–2,2 at two doses given alone or simultaneously with 20 ng E_2 were determined in the classical estrogen bioassay, uterotrophic stimulation in the immature rat (12). Twenty nanograms of E_2 is a relatively low dose, given that 5 ng E_2 is the lowest dose that consistently produces statistically significant uterotrophic stimulation (3). As shown in Fig. 5, the 1-μg dose of E11–2,2 produced a very small uterotrophic response that was not statistically different from the control. This dose of E11–2,2 partially inhibited the uterotrophic stimulation of E_2 (37%; $P < 0.001$). A larger dose (10 μg) of E11–2,2 produced a small, but statistically significant, increase in uterine weight ($P < 0.01$). However, as shown in Fig. 5, this dose of E11–2,2 completely inhibited E_2 stimulation of the uterus ($P < 0.001$), *e.g.* the uterine weight of the group given the combination of 10 μg E11–2,2 plus E_2 was the same as that of animals receiving 10 μg E11–2,2 alone. Although E11–2,2 was slightly uterotrophic, this effect, unlike that of E_2, was not due to the stimulation of cell growth (see below). E11–2,2 completely abolished the action of E_2 on the uterus.

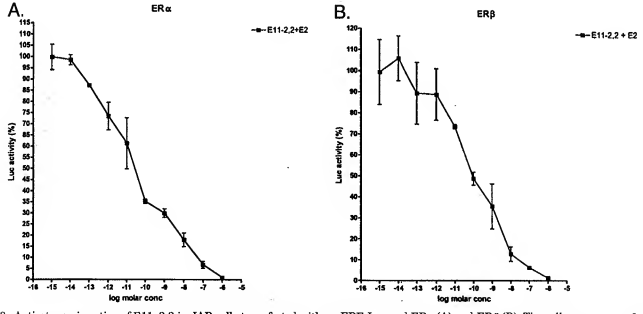

FIG. 3. Antiestrogenic action of E11–2,2 in JAR cells transfected with an ERE-Luc and ERα (A) and ERβ (B). The cells were grown for 12 h in the presence of 10^{-9} M E_2 and the indicated concentrations of E11–2,2. In both panels, 100% is the luciferase response normalized to 10^{-9} M E_2 alone. *Error bars* show the SD.

Ovariectomized rat (35-d treatment)

To test for possible tissue-selective effects of E11–2,2, body weight, uterine weight, plasma cholesterol, and various bone parameters were measured in ovariectomized adult female rats given three different doses of E11–2,2, E_2, or vehicle (sesame oil). This experimental design is similar to that previously reported for raloxifene (15), except in that study the compounds were administered orally and compared with ethinyl estradiol, whereas in this study the steroids were administered sc and compared with E_2. Oral administration was avoided because E11–2,2 is an ester and is easily cleaved by hydrolytic enzymes. It is unlikely to survive in the gastric environment. Subcutaneous administration of estrogens does not abrogate hepatic stimulation, which results in the lowering of blood cholesterol (16).

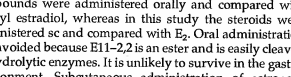

FIG. 4. Western blot analysis of ERα. Ishikawa cells were grown for 3 d with the following treatments: 1) vehicle control, 2) 10^{-8} M E_2, 3) 10^{-8} M E11–2,2, 4) 10^{-7} M E_2, and 5) 10^{-7} M E11–2,2.

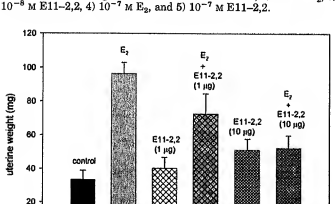

FIG. 5. Uterotrophic action of E11–2,2 in the immature rat. Twenty-one-day-old rats were injected sc (total dose) with 20 ng E_2 with or without the indicated dose of E11–2,2 in 0.1 ml sesame oil or with vehicle alone (control) for 3 d. The 1-μg dose of E11–2,2 did not stimulate the uterus, but produced a statistically significant decrease ($P < 0.01$) in E_2 stimulation. E11–2,2 at 10 μg caused a statistically significant increase ($P < 0.01$) in uterine weight, but completely inhibited the effect of E_2 ($P < 0.01$). *Error bars* show the SD.

Body weight. Compared with the ovariectomized control, only E_2 and the highest dose of E11–2,2 (600 μg/kg) produced a statistically significant decrease in body weight (Table 1). The weights of the animals treated with the two lower doses of E11–2,2 were the same as those of the controls.

Uterus. Of the three doses of E11–2,2 only two of them, the lowest and the highest, produced a statistically significant increase in uterine weight (20 μg/kg, $P < 0.01$; 600 μg/kg, $P < 0.05$; Table 1). As evident from the data in Table 1, the uterotrophic weight response to E11–2,2 was not dose dependent, because the uteri in the animals receiving the lowest dose of E11–2,2 weighed more (324 mg) than those in the animals receiving the highest dose (306 mg). The uteri from the animals receiving the intermediate dose of E11–2,2 (60 μg/kg) were not significantly different from those of the controls. None of these groups approached the uterine stimulation observed in the E_2-treated group (475 mg). In addition to uterine weight, histomorphometric analysis of the height of cells in the lumen and gland of the uterus was performed (Table 1). As shown, E_2 more than doubled cell height in the lumen, whereas the two lower doses of E11–2,2 had no effect, and the increase in cell height of the lumen in the group receiving the highest dose (600 μg/kg) was not statistically significant. In the gland, E_2 almost doubled the cell height, and again, there was no statistically significant effect of any of the doses of E11–2,2.

Plasma cholesterol levels. E_2 (400 ng/kg) decreased plasma cholesterol levels to a little more than 70% of those in ovariectomized controls (Fig. 6). Likewise, E11–2,2 decreased plasma cholesterol at the two higher doses (60 and 600 μg/kg; $P < 0.01$). The cholesterol level in the group receiving the lowest dose of E11–2,2 (20 μg/kg), although lower, was not significantly different from the control level. Compared with the animals receiving E_2, the effect of the 60 μg/kg dose of E11–2,2 was approximately the same, whereas the cholesterol level in the 600 μg/kg group was lower than that in the E_2 group ($P < 0.05$).

Bone. A large number of bone indexes were assessed in the ovariectomized rat by histomorphometric analysis. In all, eight of them showed significant ($P < 0.01$) differences between the ovariectomized controls and the E_2-treated animals, including bone volume, osteoid volume, total osteoid

TABLE 1. Effect of E11-2,2 on body weight, uterus, and bone in the ovariectomized rat

Group	Body weight (g)	Uterus			Bone		
		Weight (mg)	Lumen height (μm)	Gland height (μm)	Bone volume (%)	Trabecular space (μm)	Trabecular no/mm
Ovariectomized control	361 ± 22	195 ± 37	10.6 ± 1.9	11.5 ± 3.0	27.3 ± 3.9	79.5 ± 16.5	9.4 ± 1.4
E_2 (0.4 μg/kg)	327 ± 20[a]	475 ± 114[b]	25.4 ± 5.2[b]	18.5 ± 5.1[b]	46.0 ± 9.4[b]	38.2 ± 11.7[b]	14.6 ± 1.9[b]
E11-2,2 (20 μg/kg)	357 ± 25[c]	324 ± 8[b]	7.5 ± 1.0[c]	10.3 ± 1.4[c]	31.2 ± 3.7[c]	67.8 ± 5.5[a]	10.2 ± 0.6[c]
E11-2,2 (60 μg/kg)	360 ± 8[c]	288 ± 25[c]	10.7 ± 2.2[c]	13.0 ± 1.9[c]	31.9 ± 4.4[c]	63.1 ± 8.0[a]	10.9 ± 0.9[c]
E11-2,2 (600 μg/kg)	310 ± 15[b]	306 ± 58[c]	14.8 ± 2.6[c]	14.2 ± 1.2[c]	35.2 ± 5.3[c]	53.6 ± 7.5[b]	12.2 ± 0.8[b]

Ovariectomized rats were treated for 35 d with the indicated doses of steroids; n = 5–6. Values in the *brackets* are ±SD.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] Not statistically different from the control.

surface, osteoid surface, osteoid volume density, osteoid surface density, trabecular separation, and trabecular number. Three of them are presented in Table 1. Only the estrogen-like effect on trabecular space was statistically significant at all three doses of E11–2,2. Stimulation of trabecular number was statistically significant only at the highest dose of E11–2,2. At the two lower doses of E11–2,2, trabecular number was higher than the ovariectomized control value, but this trend was not statistically significant. The same trend was evident for bone volume with E11–2,2. Of the remaining bone indexes (above) in which E_2 produced a statistical effect at $P < 0.01$, none of them showed a statistically significant response to any dose of E11–2,2. E_2 significantly stimulated five other indexes ($P < 0.05$): osteoid volume, osteoid /lamellar osteoid surface, osteoclast surface, osteoclast bone surface density, and osteoblast surface density. Three of these five had trends with E11–2,2 similar to E_2, but none was statistically significant. In all, none of this latter group was significantly affected by E11–2,2.

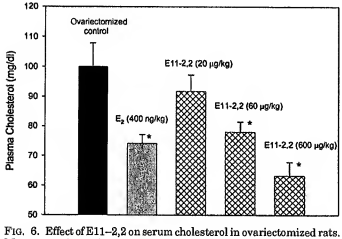

FIG. 6. Effect of E11–2,2 on serum cholesterol in ovariectomized rats. Mature ovariectomized rats were injected sc daily with E_2 or E11–2,2 at the indicated dose in 0.1 ml sesame oil for 35 d. *, $P < 0.01$. *Error bars* show the SD.

Ovariectomized rat (8-d treatment)

Another study was performed in ovariectomized rats that were treated with the highest dose of E11–2,2 used in the previous experiment (600 μg/kg) and compared with E_2-treated and control groups (Fig. 7). In this experiment, this dose of E11–2,2 again was slightly uterotrophic compared with the controls. Dry uterine weight was also slightly elevated. The increase in both uterine parameters was considerably less than that the E_2-treated animals. As previously reported, E_2 increased the core body temperature compared with the ovariectomized controls (17). The core body temperature of the E11–2,2 group was higher than that of the controls, but this was not statistically different from either the control or the E_2 groups. In contrast, although E_2 treatment lowered both body weight and plasma cholesterol levels, the effect of E11–2,2 was greater on both parameters.

Discussion

In addition to the classical ER, now termed α (ERα), another receptor has been discovered, β (ERβ) that is present in relatively large amounts in the prostate and ovary (10). Although the physiological role of ERβ is not totally clear, peripheral monocytes that express ERβ predominantly respond to estrogen stimulation by undergoing apoptosis (7), and ERβ knockout mice develop a myeloproliferative disease similar to myeloid leukemia (18). Thus, estrogens acting through ERβ influence physiological processes that are not usually associated with these ovarian hormones. It is re-

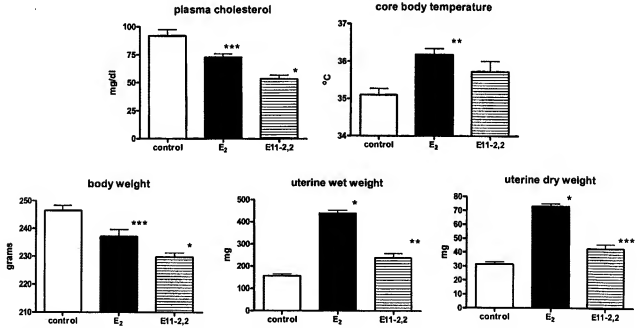

FIG. 7. Effect of E11–2,2 (600 μg/kg) and E_2 (400 ng/kg) treatment for 8 d in ovariectomized rats. *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$. *Error bars* show the SD.

ported (patent application) that 11β-analogs of E_2, which are similar in substituent length to E11–2,2, but substituted instead with pure alkanes and alkenes from C_5 to C_9 in length, are antagonists with ERβ, but agonists with ERα (19) in cells transfected separately with the two ER subtypes and a reporter gene. If E11–2,2 acts through the same mechanism, its antiestrogenic effect would be transmitted through ERβ. However, it seemed likely to us that the inhibitory effect of E11–2,2 on Ishikawa cell AlkP is different and that it is directed through ERα. Although the Ishikawa cell contains both ERα and ERβ (20), various ligands, such as 16α-substituted estradiol analogs (3, 5), which bind to ERα preferentially (21), produce a stimulatory effect on Ishikawa cell AlkP appropriate to their ERα affinity (3), whereas phytoestrogens, which bind to ERβ preferentially (22), produce a poor response (6, 23). We found that the pure ERα agonist, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (24), is very potent in the stimulation of Ishikawa cell AlkP and that the ERβ agonist 2,3-bis(4-hydroxyphenyl)propionitrile (25) is almost 500-fold less active (unpublished observations). This strongly suggests that Ishikawa AlkP stimulation acts preferentially through ERα. Nevertheless, because E11–2,2 binds to ERβ with high affinity (4), and the two ER subtypes can form heterodimers (26), the possibility existed that the antiestrogenic action of E11–2,2 could be caused by the formation of an inhibitory complex, a heterodimer, ERβ-E11–2,2/ERα-E_2, and also, if E11–2,2 is an ERα agonist, the heterodimer ERβ-E11–2,2/ERα-E11–2,2. Consequently, we investigated the action of E11–2,2 in JAR cells that had been separately transfected with ERα and ERβ and an ERE linked to a Luc reporter gene. E11–2,2 inhibits E_2-stimulated transcription of the reporter gene with both ER subtypes. Clearly, if the E_2 11β-substituted alkanes and alkenes (above) are ERα agonists and, conversely, ERβ antagonists, then E11–2,2 which is an ERα antagonist, is acting through a markedly different mechanism with ERα.

The antiestrogenic action of E11–2,2 was investigated further in the classical estrogen bioassay by determining its effect on the uterotrophic stimulation of E_2 in the immature rat, an ERα model. The rat uterus has a preponderance of ERα compared with ERβ (23), and the uterotrophic response to estrogens has been shown to be ERα ligand selective (27). Additionally, the uterus of the ERα knockout mouse does not respond to E_2 (28). As shown in Fig. 5, E11–2,2 inhibits the uterotrophic stimulation of E_2. Clearly, E11–2,2 possesses antiestrogenic action in the uterus, an ERα-selective action.

The slight stimulatory activity of E11–2,2 alone in the Ishikawa assay and the uterotrophic assay more closely resembles that produced by SERMs rather than that of pure antiestrogens. which have no stimulatory action (29). This was tested *in vivo* in the ovariectomized rat. As in the immature rat, E11–2,2 again caused a slight increase in uterine weight. However, this was not dose dependent, and the small stimulation seen in the group receiving the intermediate dose (60 μg/kg) was not statistically different from that in the ovariectomized control. Moreover, histomorphometric analysis showed that the uterus in E11–2,2-treated animals was not different from that in the control at any of the doses. One likely explanation for the increase in uterine weight in the E11–2,2 groups that is not paralleled by an increase in cell size is that the weight gain is due predominantly to water imbibition, which has been shown to occur through a pathway distinct from that of classical estrogen action (30). However, uterine dry weight is elevated over the control group in the animals treated with 600 μg/kg E11–2,2 for 8 d, which eliminates this possibility. A more likely explanation is that the difference in uterine weight is too small to be detected morphologically. In contrast to the uterus, E11–2,2 produces a dose-dependent decrease in plasma cholesterol. The decrease in plasma cholesterol is a well known estrogenic action on the liver, which in the rat causes an increase in hepatic low density lipoprotein receptors with a concomitant clearance of low and high density lipoprotein cholesterol (31). Thus, in contrast to the uterus, E11–2,2 induces an estrogenic effect in the liver. Although E11–2,2 has some effect on the bone (Table 1), it is modest. In the 8-d study, the same effect of E11–2,2 on body weight and cholesterol level was observed. In this experiment the estrogenic effect on core body temperature was measured, and although E_2 increased the temperature significantly, the rise observed with E11–2,2 was not statistically significant. The cause of this weak effect in bone and body temperature is not known, but some of the possibilities are: tissue-specific transcriptional activity, low ER levels, or local metabolic inactivation of this labile steroid. These studies demonstrate that E11–2,2 has the properties of a SERM: it is antiestrogenic in the Ishikawa cell, in JAR cells transfected with ERα or ERβ, and *in vivo* in the uterus, but it is estrogenic in liver and bone.

For all antiestrogens (ERα), the key structural feature in their biological action is their long and polar side-chain. It prevents helix 12 in the ER from attaining the conformation required for coactivator binding, which leads to gene transactivation (32). As shown in Fig. 1, the difference between E11–2,2 and other antiestrogens is not subtle. The side-chain is uncharged, nonpolar, and small compared with the other antiestrogens. The ester group in the side-chain of E11–2,2 is present in exactly the same position and at the same distance from the steroid nucleus as it is in E11–2,1, which is a potent estrogen agonist. Thus, the ester group by itself is obviously not the cause of the antiestrogenic action. Structurally, the difference between the estrogen E11–2,1 and the antiestrogen E11–2,2 is minor, a single methylene group. This difference between a methyl ester and an ethyl ester increases the length of the side-chain by one carbon atom, from four to five nonhydrogen atoms. This seemingly small difference produces a dramatic change from an estrogen to an antiestrogen. Small structural modifications such as this that lead to large biological changes are called an activity cliff (33). Apparently, the increase from four to five atoms in the 11β side-chain produces an activity cliff in the interactions of E11–2,1 and E11–2,2 with the ER. We found that moving the ester group further from the ring by increasing the carboxylic acid group by one methylene unit, as in E_2-11β-yl-propionate, has little effect on ER binding of the methyl (E11–3,1) or ethyl (E11–3,2) esters. Both have high affinity for ER, and again, both are almost devoid of estrogenic activity in the Ishikawa assay (4). Like E11–2,2, these two E_2 analogs are potent antiestrogens in the Ishikawa cell assay (not shown). Thus, the changeover to an antiestrogen is not simply due to the difference between a methyl and an ethyl ester, nor is it re-

stricted to a unique length of five atoms. In the latter compound, E11–3,2, the length of the side-chain is six (nonhydrogen) atoms (five carbons and one oxygen). Consequently, it appears that five atoms is the minimum length, but not the exclusive length, that is required for the side-chain to impart antiestrogenic properties to the steroidal structure.

The x-ray crystal structure of the ER ligand binding domain (LBD) in complex with several agonists and antagonists has been analyzed (32, 34). Comparisons of the conformations of these structures have provided strong evidence of the mechanisms involved. The binding of an agonist to ER involves a complicated complex of several different regions and helixes of the LBD. Similar conformational changes occur upon binding of various antiestrogens and SERMs. However, with these ligands, helix 12, the most C-terminal helix in the LBD, does not attain the proper orientation. The long side-chain present in SERMs extends out of the ER binding pocket and prevents helix 12 from assuming the agonist-activated conformation (32, 34). It assumes an alternate conformation, which prevents binding to the ER of various coactivators that are necessary for transcriptional activation (32, 34). Pure antiestrogens act through a different, but related, mechanism. The crystal structure of the pure antiestrogen, ICI 164,384, bound to the LBD of ERβ has been determined, and in this complex the side-chain protruding from the steroid nucleus at C-7α also prevents helix 12 from attaining its proper orientation (35). The side-chain of ICI 164,384 binds directly to the coactivator recruitment site of the LBD and physically stops the agonist positioning of helix 12. This results in the destabilization of helix 12, which leads to proteolysis of the antiestrogen-ER complex and, thus, depletion of cellular ER (36). As would be expected for a SERM, E11–2,2 had no effect on the concentration of ERα in Ishikawa cells.

If the long and polar side-chain is the critical factor in blocking estrogen gene activation, how does a steroid with a relatively small and nonpolar side-chain, such as E11–2,2, function as an antiestrogen (SERM)? Given the structures of the other antiestrogens, it is likely that E11–2,2 does not have the characteristics that are required to prevent helix 12 from adopting the ligand-activated conformation. As noted above, E_2 analogs with 11β side-chains consisting of alkanes and alkenes five carbon atoms long (E11–5) or longer, compounds similar to E11–2,2, have been reported to be estrogen antagonists, but only with ERβ (19). In contrast to E11–2,2, these are ERα agonists. This differential behavior between the two ER subtypes is not unprecedented. The R,R enantiomer of tetrahydrochrysene (R,R-THC) has two ethyl groups as side-chains, and it, too, is an ERβ antagonist and an ERα agonist (37). The crystal structure of the THC-ERβ complex has been determined and found to be very different from that produced by the interaction of the long side-chain antagonists (38). THC, lacking a bulky side-chain, cannot prevent helix 12 in ERβ from adopting the agonist-activated conformation; instead, it appears to stabilize helix 12 in an inactive conformation. Thus, the mechanism of THC estrogen antagonism of ERβ has been termed passive antagonism, differentiating it from antiestrogenic mechanisms that directly prevent helix 12 agonist positioning, termed active antagonism. Perhaps the 11β-pentyl analog of E_2, E11–5, which also has a short side-chain, acts as ERβ-antiestrogens through a passive mechanism.

Obviously, there are strong similarities between E11–2,2 and E11–5. That E11–2,2 is an antagonist of ERα, whereas E11–5 is an agonist, strongly suggests that the oxygen atoms in the ester group are responsible for this difference, possibly through hydrogen binding to amino acids in either helix 12 or to the groove in the LBD to which helix 12 must bind in the agonist state. Currently we are performing structure-activity relationship studies to determine the specific structural requirements that are necessary for ERα antagonism. It should be possible to design SERMs that are similar to E11–2,2, but contain metabolically stable substituents. Importantly, such estrogenic ligands with apparently different mechanisms of antagonism could exert novel biological and therapeutic effects (38).

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